PSORIASIS ACTIVATION OF CELLS IMPORTANT IN CARDIOVASCULAR DISEASE

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Abstract

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Psoriasis is an immune mediated inflammatory disease which affects 2-3% of the world's population. Over the last decade, psoriasis has been acknowledged as an independent risk factor for atherosclerosis. The precise mechanism or mechanisms of the heightened risk is widely speculated. Endothelial cells and macrophages are central players in the immunopathological development of both diseases.

Interleukin-36 cytokines (IL-36) have been heavily implicated in psoriasis immunopathology. Significant upregulation of epidermal IL-36 is a recognised characteristic of psoriatic skin inflammation. IL-36 induces inflammatory responses in dendritic cells, fibroblasts and epithelial cells. While vascular alterations are a hallmark of psoriatic lesions and dermal endothelial cells are well known to play a critical role in dermal inflammation, the effects of IL-36 on endothelial cells have not been defined.

We report that endothelial cells including dermal microvascular cells express a functionally active IL-36 receptor. Adhesion molecules VCAM-1 and ICAM-1 are upregulated following IL-36γ stimulation, and this is reversed in the presence of the endogenous IL-36 receptor antagonist. IL-36γ-stimulated endothelial cells secrete the proinflammatory chemokines IL-8, CCL2 and CCL20. Chemotaxis

assays showed increased migration of T-cells following IL-36γ stimulation of endothelial cells.

Both resident and infiltrating inflammatory myeloid cells contribute to the immunopathology of psoriasis by promoting the IL-23/IL-17 axis. We show that IL-36γ induces the production of psoriasis-associated cytokines from macrophages (IL-23, TNFα) and that this response is enhanced in macrophages from psoriasis patients. This effect is specific for IL-36γ and could not be mimicked by other IL-1 family cytokines such as IL-1α. Furthermore, IL-36γ stimulated macrophages potently activated endothelial cells as illustrated by ICAM-1(CD54) upregulation, and led to increased adherence of monocytes, effects that were markedly more pronounced for psoriatic macrophages. Interestingly, regardless of stimulus, monocytes isolated from psoriasis patients showed increased adherence to both the stimulated and unstimulated endothelium when compared to monocytes from healthy individuals.

Collectively, these findings add to the growing evidence for IL-36γ having roles in psoriatic responses, by enhancing endothelium directed leukocyte infiltration into the skin and strengthening the IL-23/IL-17 pathway. Our findings also point to a cellular response which could potentially support cardiovascular comorbidities in psoriasis.

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Published Papers

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Published abstracts

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Bridgewood, C., et al. "The novel cytokine interleukin-36 has proinflammatory effects on human endothelial cells." ***British Journal of Dermatology*** 174.5 (2016): e67.

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Submitted papers

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1) Introduction

Psoriasis is an autoimmune disease that causes plaques of red raised scaly skin. This condition affects around 2-3% of the world population and is a chronic relapsing inflammatory disease (Gelfand et al., 2005). The most common form of the disease is psoriasis vulgaris which is identifiable by papulosquamous plaques on the body (Raut et al., 2013). However, psoriasis is not just associated with the skin; it is a systemic disease and it is linked to other conditions such as diabetes and metabolic syndrome (Sommer et al., 2006). Overall, there is evidence that people with psoriasis have a reduced quality of life (de Korte et al., 2004, Krueger et al., 2001) but are provided with a variety of treatments available to ease symptoms, however the underlying causes of psoriasis are yet to be fully deciphered.

A wide range of treatments are available for treating psoriasis, including, topical, systemic and phototherapy. Treatments are chosen based on disease severity, patient preference, cost, efficacy and the response of the individual patient (Menter and Griffiths, 2007, Feldman et al., 2003). Patients are often grouped into to 'mild to moderate' or 'moderate to severe' with respect to psoriasis severity. Mild to moderate psoriasis symptoms can often be manged with topical agents such as corticosteroids and emollients (Feldman, 2016, Freedman et al., 1963). However moderate to severe psoriasis often requires systemic agents. Systemic agents are often defined as biologic and non-biologic. Immunosuppressive or immunomodulatory include methotrexate and cyclosporine. Biologics work by targeting a specific part of the immune system

(e.g. cytokines), examples of biologics used for treating moderate to severe psoriasis include, adalimumab, etanercept, infliximab, ustekinumab and secukinumab (Feldman, 2016, Menter et al., 2008, Mease et al., 2000, Reich et al., 2005, Papp et al., 2008, Langley et al., 2014). Various phototherapy treatments are also available which can be used in combination or alone with the above.

Atherosclerosis is a cardiovascular chronic inflammatory disease which results in plaque formation at particular points in the arteries, reducing blood flow to the vital tissues such as the heart and brain. These plaques can become unstable and rupture causing myocardial infarction or stroke. 160,000 people a year die in the UK alone from Cardiovascular Disease (CVD). Atherosclerosis and cardiovascular disease (CVD) represent a major burden on healthcare. In 2012/13 the cost of treating CVD was 6.8 billion which accounts for 7% of NHS expenditure (British Heart Foundation).

It has been over 30 years since a potential link between psoriasis and developing CVD was first proposed (McDonald and Calabresi, 1978). Evidence now suggests that psoriasis is an independent risk factor for developing CVD (Gelfand et al., 2006, Naik et al., 2015, Fang et al., 2016, Dey et al., 2017, Egeberg et al., 2017, Bissonnette et al., 2017). However, psoriasis is often co-incident with other cardiovascular risk factors such as smoking, diabetes, obesity and hypertension, so identifying psoriasis as an independent risk factor has been refuted by some groups (Neimann et al., 2006, Dowlatshahi et al., 2013, Stern and Huibregtse, 2011, Parisi et al., 2015).

Psoriasis patients do not have separate CVD screening unless they present with traditional risk factors (Kimball et al., 2012, Parsi et al., 2012). If psoriasis is an independent risk factor for CVD, this could mean treating the traditional risk factors such as smoking and obesity do not go far enough. Some researchers now believe that a CVD preventative regimen needs to be developed for severe psoriasis patients and that early CVD detection and monitoring in these patients must be improved (Coumbe et al., 2014).

Another area of concern for psoriasis patients is regarding current therapies and their possible link to CVD. Patients with moderate to severe psoriasis are often prescribed systemic therapies which may have positive or negative effects on CVD. There is a lack of randomized controlled trials (RCTs) which directly relate psoriasis treatments to potential effects on CVD, thus meaning the true effects of such treatments which control psoriasis symptoms on vascular inflammation are unknown.

Anti-inflammatory treatments such as tumour necrosis factor-α (TNFα) inhibitors may in fact lower cardiovascular risk (Dixon et al., 2007, Nguyen and Wu, 2014). IL-17 inhibitors used for psoriasis could also potentially exacerbate or decrease the atherosclerosis risk (Gong et al., 2015). Statins used to treat CVD may have anti-inflammatory effects that are beneficial to psoriasis (Weitz-Schmidt et al., 2001). However, beta-blockers which are used to treat high blood pressure have been shown to induce psoriasis (Abel et al., 1986).

RCTs are currently in progress for CVD and psoriasis treatments such as inhibitors for TNFα, IL-17 and IL-23 (Takeshita et al., 2017). A more detailed

description of these cytokines and their potential inhibitors will be given in later chapters.

With psoriasis emerging as an independent risk factor for CVD, research has started to focus on potential common mechanisms between the two diseases at a molecular level. The majority of research so far focuses on shared inflammatory molecules and pathways between the diseases. This introduction will highlight the central role of endothelial cells (ECs) in both diseases that could cause dysfunction. ECs in the vessel wall are key to atherogenesis, being the site at which inflammatory pathways are stimulated, chronic inflammation then results in continued immune cell recruitment into the subendothelium and remodelling (Williams and Tabas, 1995). In common with atherosclerosis, in psoriasis ECs also play a role as angiogenesis and vascular remodelling occurs at plaque sites. Several important questions are at present unanswered but could have major implications to improve treatments. Are the vascular ECs from psoriasis patients more prone or predisposed to inflammation and thus promote atherosclerosis? How do ECs respond to the systemic effects of psoriasis? These answers could add to the evidence that psoriasis is an independent risk factor to CVD and help with guidelines for future treatments for psoriasis patients and also identify possible therapeutic targets. The introduction will consider the current literature on this topic, after providing an introduction to both diseases and the immunology and mechanisms behind them.

1.1 Atherosclerosis- mechanisms and immunology

Atherosclerosis is a chronic inflammatory disease that manifests in specific regions of low or disturbed flow within the arteries and results in plaque formation

and tissue remodelling. The build-up of these plaques and reduction of blood flow can cause adverse cardiovascular events such as stroke (Faxon et al., 2004). Around 30% of deaths in the USA are caused by CVD and over 50% of these deaths are directly linked to atherosclerosis (Roger et al., 2012). It is now acknowledged that atherosclerotic lesions develop at arterial branches and sites that are subject to lower blood flow and thus less shear stress (Heo et al., 2014).

Before any signs of the disease are present, the endothelial regions within arteries that are prone to atherosclerosis (low or disturbed flow) show functional changes (cellular alignment and enhanced adhesiveness to circulating blood cells) associated with increased activation of both the mitogen-activated protein kinase (MAPK) pathway and nuclear factor kappa-B (NF-κB) pathway (Passerini et al., 2004, Hajra et al., 2000). This in turn increases expression of leukocyte adhesion molecules on the endothelial cell surface such as E-selectin, ICAM-1 and VCAM-1 which encourage capturing, rolling and adhesion of leukocytes (Blankenberg et al., 2003).

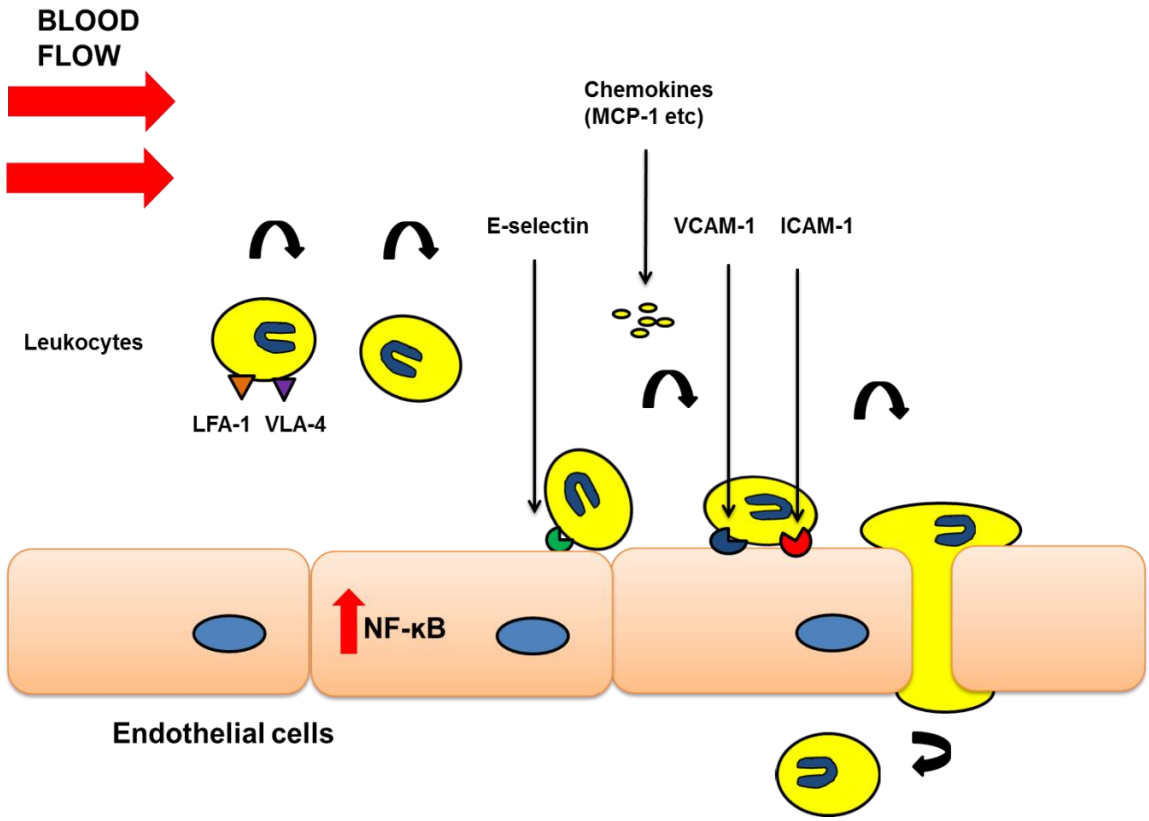


Figure 1: Leukocyte adhesion to the endothelium

Inflammatory activated pathways such as NF-κB lead to increased adhesion molecule expression in ECs. Selectins encourage leukocyte capture and rolling on the vessel wall. Chemokines such as IL-8 and MCP-1 facilitate integrin activation on leukocytes, which allows integrin-adhesion molecule interactions to take place. The leukocytes which have adhered are able to transmigrate and reach the underlying tissue. Adapted from (Warboys et al., 2011). VCAM-1: Vascular cell adhesion molecule 1, ICAM-1: Intercellular Adhesion Molecule 1, MCP-1: Monocyte chemotactic protein 1.

Junctions between activated ECs are leaky and blood lipids such as low-density lipoproteins (LDL) begin to accumulate at the tunica intima and have greater chance of being oxidised (Steinberg, 1997, Steinberg et al., 1989). One of the

first identifiable features of atherosclerotic plaques are fatty streaks; these contain lipid loaded macrophages and a smooth muscle layer (Stary et al., 1994). These lesions are caused by monocytes that travel to the tunica intima and then differentiate into macrophages, ingest oxidised lipids and become foam cells (fat laden macrophages) (Stary et al., 1994). A positive feedback loop is then created when foam cells secrete proinflammatory cytokines, which act further on the ECs and recruited monocytes to promote endothelial activation (van der Wal et al., 1994). The elastic lamina (separates the smooth muscle layers from the endothelium) is also degraded as a result of this inflammation, causing smooth muscle cells (SMCs) to migrate towards the sub-endothelium. The SMCs then produce collagens that cover the plaque in a fibrous capsule thickening the arterial wall (Ang et al., 1990). Despite this outward remodelling, the plaque eventually begins to encroach the lumen, which limits blood flow. Cells in the centre of the plaque begin necrosis at a quicker rate than they can be cleared, which creates a necrotic core causing further inflammation. Finally, if the fibrous cap ruptures, this can cause blockage and fatal cardiovascular events (Fuster et al., 1992).

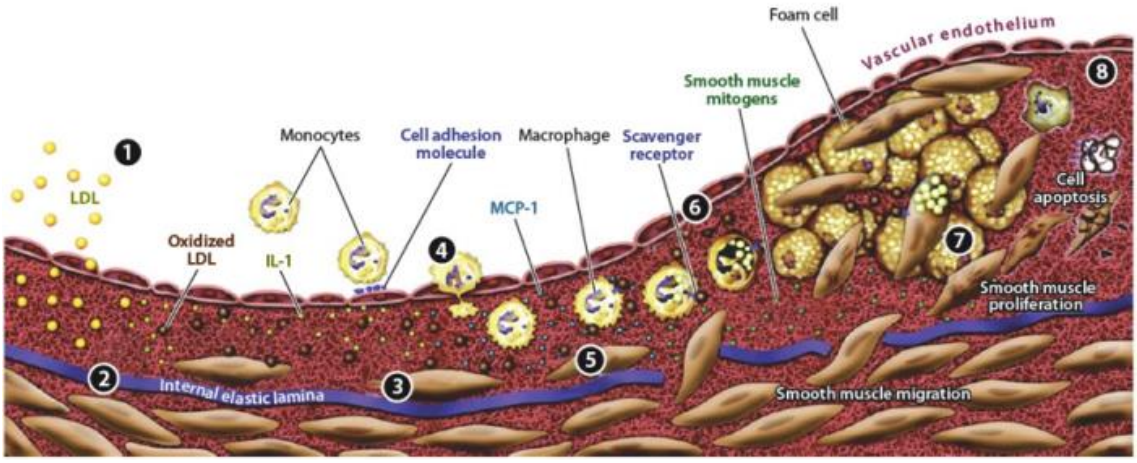


Figure 2: Initiation stage of atherosclerosis

Stages 1-2, LDL move into the sub endothelium. Monocytes move in aided by cytokines and adhesion molecules (4). Macrophages ingest LDL and become foam cells (6-7). SMCs and foam cells accumulate and cause the plaque (7-8). Taken from (Faxon et al., 2004).

1.2 Psoriasis

Psoriasis is a common skin disease that affects 2-3% of the world population. The most common type, psoriasis vulgaris, is easily diagnosed by its red erythematous plaques and silver dry scales, often found on elbows and knees (Figure 3). Other forms of psoriasis include guttate, inverse, pustular and erythrodermic. Psoriasis lesions are histologically distinct from other skin diseases, with epidermal hyperplasia and are heavily infiltrated by leukocytes. In around 30% of patients more than 10% of the body is covered and this is classed as moderate to severe psoriasis. For classing disease severity, the PASI index (Psoriasis activity and severity index) is used, which takes into account redness and thickness of plaques and area covered.

Figure 3: Psoriasis plaques

Example of psoriasis vulgaris, also known as plaque psoriasis, covering the body. Image taken from (Zangeneh and Shooshtary, 2013).

1.2.1 Features of a healthy epidermis

The outermost layer of the skin, the epidermis is a stratified squamous layer epithelium. The cell type that accounts for the majority of the epidermis is the keratinocyte. Keratinocytes are further organised into four different layers (Figure 4). The basal layer contains epidermal stem cells which have a role in maintaining a constant renewal of cells. Keratinocytes migrate through the different cell layers and eventually begin to differentiate. Keratinocytes lose their nuclei and become corneocytes and these are eventually shed. The process from the basal layer to the shedding of corneocytes takes around one month. The dermis is located below the epidermis and this contains collagen and elastic tissue, but also capillaries which function to deliver nutrition and oxygen to the skin (Pastar et al., 2014, Takeo et al., 2015).

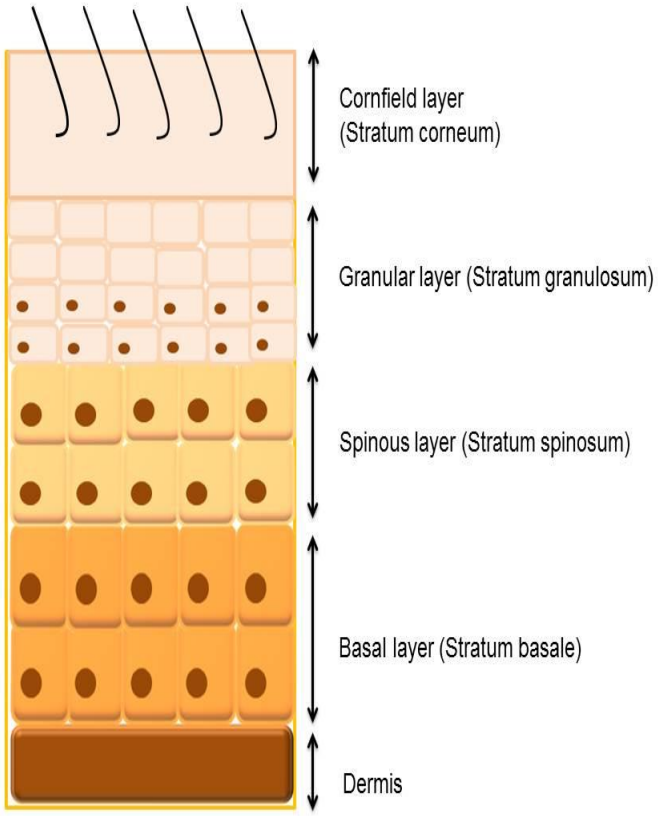


Figure 4: Layers of the skin

In the basal layer, stems cell division results in a constant renewal of the layer. Keratinocytes migrate through both the spinous and granular layers. Following this, keratinocytes differentiate and lose their nuclei and are termed corneocytes.

1.2.2 Vasculature of the skin

The blood vessels within the skin comprise of both cellular and non-cellular elements such as EC and also a basement membrane consisting of smooth muscle cells and pericytes (Braverman, 1989). The blood vessel organisation within the dermal compartment is comparable to other organs. Feeder arterioles penetrate deep from the muscle. These feeder arterioles serve to supply blood to the both the superficial and dermal papilla. The superficial arterioles then

further network into to feed into dermal papillary loops (Braverman, 1989). These papillary loops represent the main site for exchange of oxygen and nutrients (Swerlick, 1997).

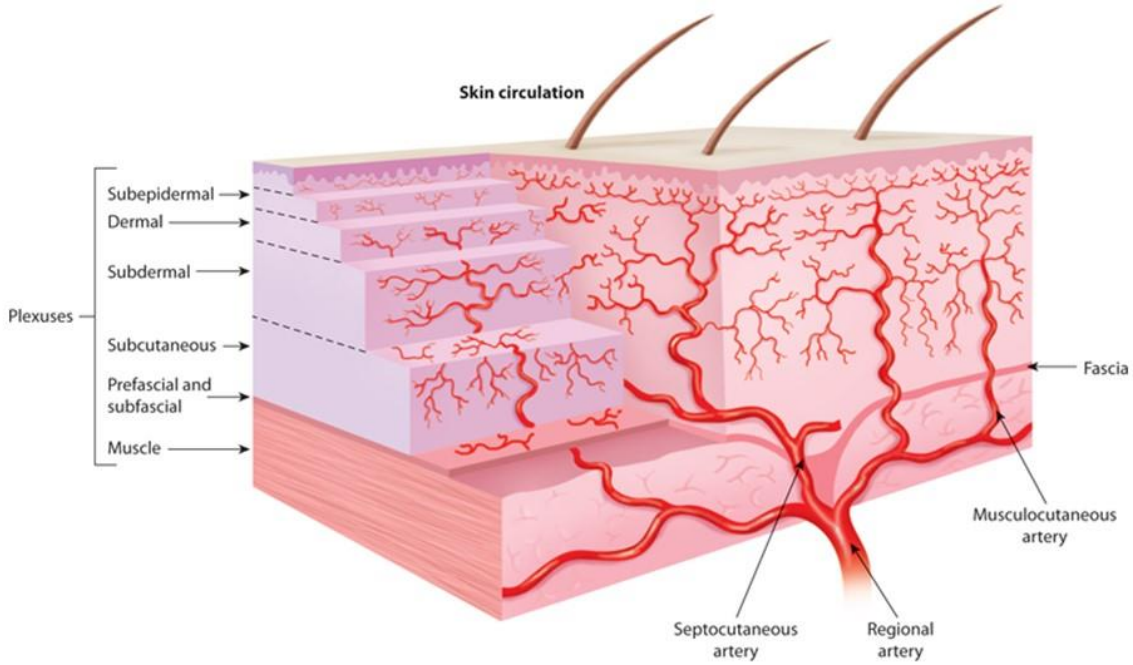


Figure 5: The dermal vascular system

Organistion of the vessels within the different compartments of the skin. Used with permission from (Hamm, 2014).

1.2.3 Features of psoriasis epidermis

A key feature in psoriatic skin is epidermal thickening; this is due to keratinocyte hyperproliferation (Bata-Csörgö and Szell, 2012, Saiag et al., 1985) (Figure 6). Keratinocyte differentiation occurs faster and the granular layer is severely reduced. Psoriatic plaques are notable for their silvery scale appearance, caused

by hyperproliferation of epidermal keratinocytes, which have a quicker process of maturation and incomplete cornification (Bata-Csörgö and Szell, 2012). Parakeratosis is also seen in the psoriatic epidermis and is characterised by the retention of nuclei in stratum corneum and causes thinning of the granular layer (Cox and Watson, 1972). The keratinocytes migration from the basal to the cornified layer usually takes a month, however in psoriasis this can occur in one week (Halprin, 1972). Unlike the healthy epidermis, the psoriatic epidermis is subject to inflammation and is associated with an increase in the number of inflammatory cells. The red appearance of psoriasis lesions is due to the increased angiogenesis of capillaries which increases the circulation in the upper dermis (Lowes et al., 2014, Hern et al., 2005).

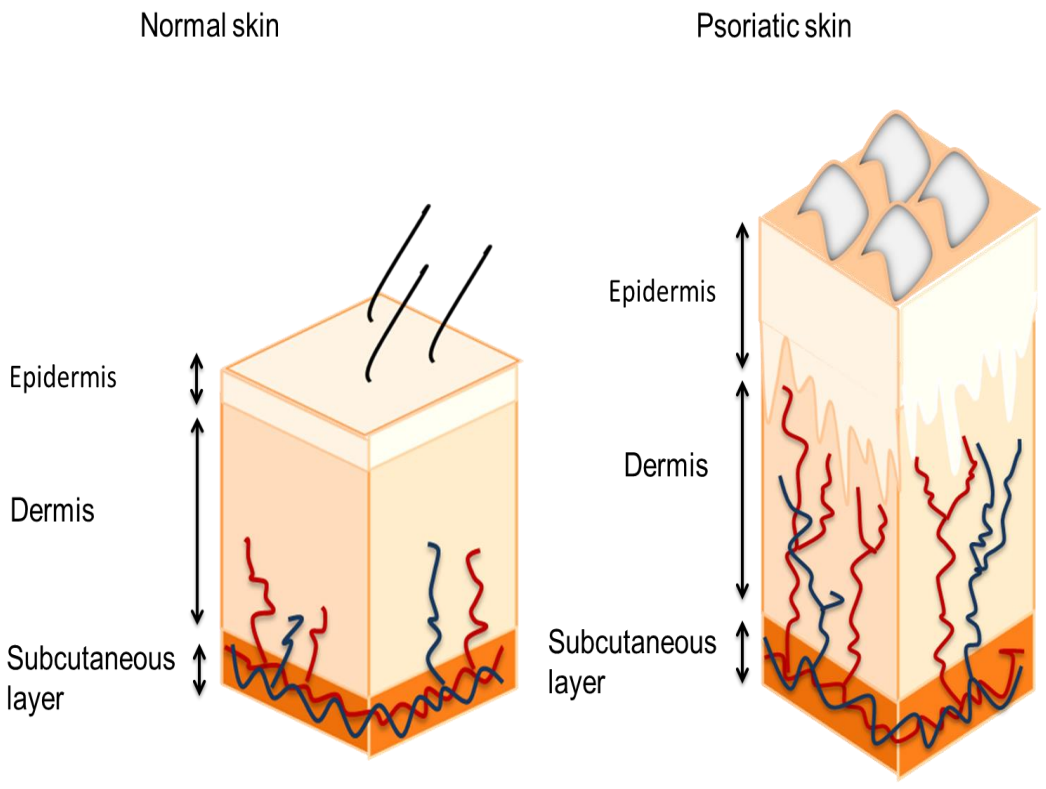


Figure 6: Cross section depiction of psoriatic skin

In psoriatic skin, the epidermal thickening is caused by hyperproliferation of keratinocytes and subsequent silver scale development. The upper

dermis of psoriatic skin contains capillaries that are both tortuous and leaky.

1.2.4 Psoriasis genetics

Psoriasis is a multi-factorial disease. Various genes and environmental triggers interact to cause the disease. Psoriasis is linked to strong genetic susceptibility, but despite this, a gene combination has never been clearly identified. Multiple genetic studies have identified susceptibility loci. Twelve different chromosomal loci (PSORS1-12) have been identified as being linked to psoriasis. Histocompatibility complex antigens are associated with psoriasis of which the most associated is HLA-Cw6 within PSORS1 (Tiilikainen et al., 1980). A detailed review of psoriasis genetics has recently been published and so is not considered further in this thesis (Harden et al., 2015).

1.2.5 Inflammatory features of psoriasis and psoriatic proinflammatory cytokines

Psoriasis immunology is highly complex process incorporating many different mechanisms involving keratinocytes, several types of leukocytes and the endothelium (Lowes et al., 2007). Early research suggested that the disease was a result of keratinocyte proliferation and differentiation (Barker, 1991). While this does occur, it is now clear that psoriasis is an immune mediated disease. Before the epidermal changes are apparent, proinflammatory infiltrates appear (Kim and Krueger, 2015). The constant over expression of cytokines is thought to be the primary cause of the initiation, maintenance and recurrence of the psoriasis plaques. Initial research into psoriasis suggested that the disease was driven by

Th1 cells and subsequent Interferon gamma (IFNγ) secretion (Schlaak et al., 1994). However, a role for Th17 cells has since emerged. IL-23, a cytokine involved in Th17 development, as well as Th17 secretary cytokines, IL-17 and IL-22, have all have been found to have major roles in psoriasis immunopathology (Meglio and Nestle, 2010, Hao, 2014, Ma et al., 2008, Lowes et al., 2008).

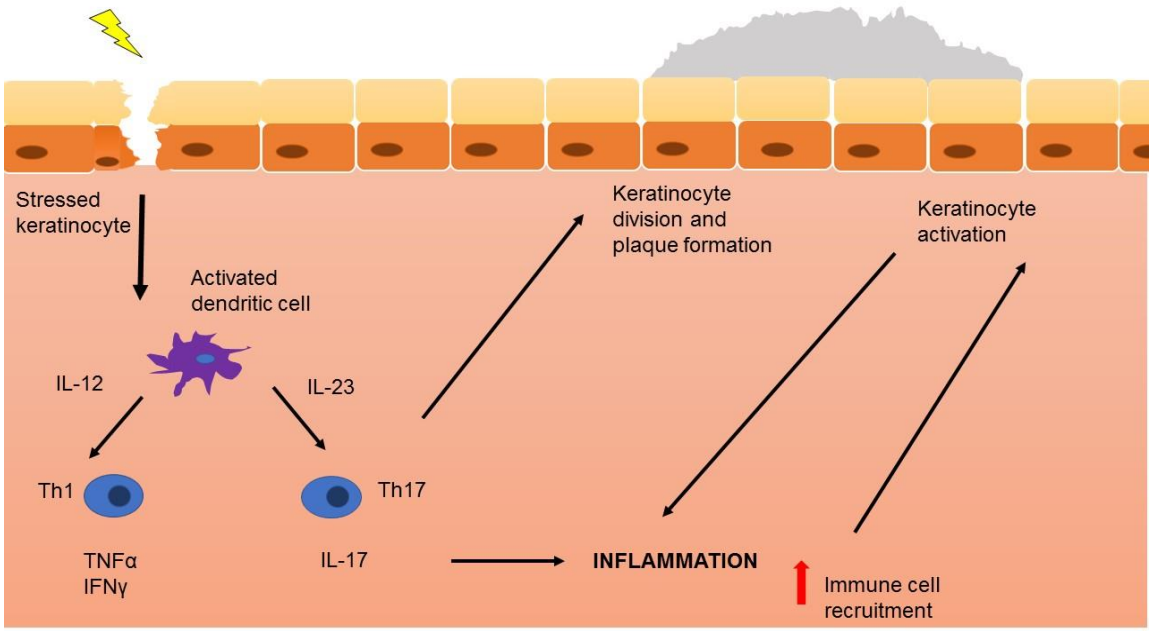


Figure 7: Psoriasis immunopathology

Trauma to the skin results in activated dendritic cells, which secrete IL-12 and IL-23 to polarize naïve Th0 cells to Th1 and Th17 cells respectively. Resultant cytokine secretion results in inflammation, immune cell recruitment and further keratinocyte activation and plaque formation.

A number of proinflammatory cytokines are involved in both the initiation and maintenance of psoriasis. IL-6, IL-23, IL-8, IL-17, IL-1, IL-36 and TNFα have all been documented, and recently growing attention has focussed on IL-19, IL-37, IL-20, IL-19 and IL-22 (Sticherling et al., 1999, Grossman et al., 1989, Witte et

al., 2014, Teng et al., 2014). Biological treatment which targets both IL-23 and IL-17 have shown greatest efficacy for treating severe psoriasis, which highlights the importance of the IL-23/IL-17 axis in the disease.

A review of cytokines and their potential role in psoriasis can be found here (Baliwag et al., 2015). IL-36 is becoming much more implicated in the recent literature.

1.3 The IL-23/IL-17 axis in psoriasis

Psoriasis development is thought to be a result of an inflammatory response in the skin, which can be triggered by injury, infection, allergy or stress. Cells of the innate immune system such as Langerhans' cells become activated (Xiao et al., 2017). Langerhans' cells which are antigen presenting cells engulf antigens or pathogens and migrate to the lymph nodes. The processed peptide antigens are then presented to T cells (Harden et al., 2015, Lowes et al., 2014). Both local myeloid dendritic cells and Langerhans cells in the lymph node that have been activated secrete IL-23, a cytokine required for Th17 maintenance (Singh et al., 2016). Naive CD4+ T (Th0) cells differentiate into Th17 cells and migrate back to the epidermis where they secrete IL-22 and a range of IL-17 family cytokines to drive psoriatic inflammation (Lowes et al., 2014)

1.4 IL-23

IL-23 is a heterodimeric cytokine produced by inflammatory myeloid cells and plays a central role in T helper 17 (Th17) responses (Iwakura and Ishigame, 2006, Yen et al., 2006). As discussed previously, the IL-23/IL-17 axis plays a central role in psoriasis and other chronic inflammatory diseases such as Crohn's

disease and rheumatoid arthritis (Siakavellas and Bamias, 2012, Lubberts, 2015). The pathogenicity of IL-23, is largely dependent on the resulting dysregulated IL-17 secretion from Th17 cells. Antagonists to both IL-17 and IL-17R in varying stages of phase development have shown anti-psoriatic efficacy (Wasilewska et al., 2016). Similar positive results have been reported with IL-23 antagonists. A detailed clinical discussion of current and phased antagonists that target these cytokines has recently been published (Campa et al., 2016).

IL-23 consists of the IL-23p19 subunit paired with the IL-12p40 subunit, the latter being shared with IL-12 (Gaffen et al., 2014). IL-23 signals through a receptor complex consisting of the IL-23 receptor (IL-23R) and IL-12Rβ1 (also shared with IL-12) (Gaffen et al., 2014) (Figure 8). IL-23 targets T cells and activates both JAK and STAT signalling molecules with a particular emphasis on STAT3 (Cho et al., 2006). While IL-12 directly promotes T cell differentiation into Th1 cells, IL-23's relationship with Th17 development is more complex. It is thought is not to be required in early Th17 development, but to play a role in terminal differentiation of Th17 cells (Bettelli et al., 2006, Mangan et al., 2006).

The immunogenetic link between IL-23 and psoriasis has gained attention in recent years. SNPs for both IL-23 subunits and also the IL-23R are associated with psoriasis (Nair et al., 2008, Cargill et al., 2007, Nair et al., 2009). The most commonly associated SNP for the IL-23R in psoriasis, encodes a R381Q amino acid substitution, where the rarer Q allele results in decreased IL-23 signalling and is thought to be thus protective against numerous autoimmune disorders such as psoriasis (Di Meglio et al., 2011).

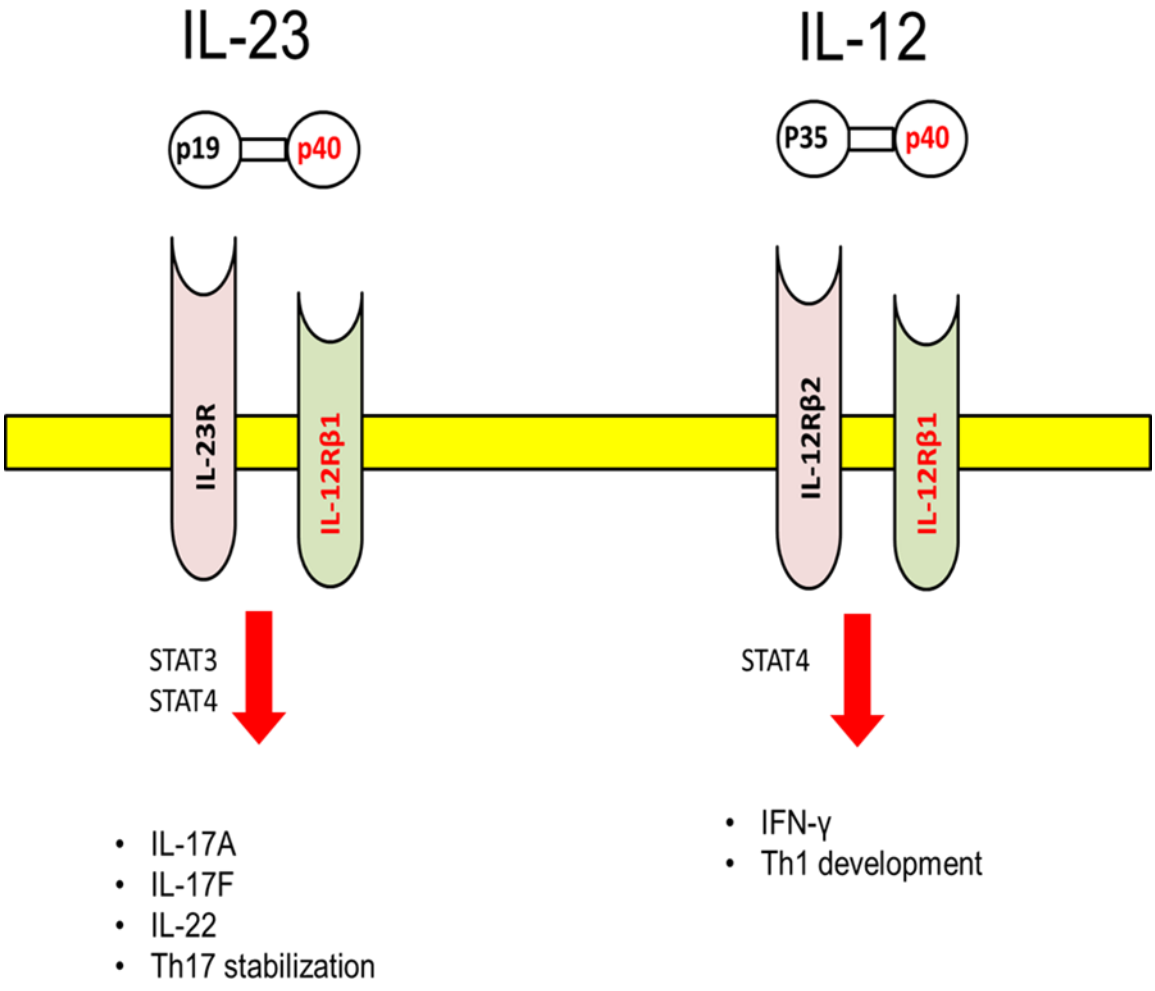


Figure 8: IL-23 and IL-12 signalling

IL-23 cytokine consists of IL-23p19 and IL-12p40 subunits. IL-23 binds to IL-23R and IL-12Rβ1. STAT3 and STAT4 signalling results in IL-17 and IL-22 secretion and stabilisation of the Th17 phenotype. IL-12 consists of the IL-12p35 and IL-12p40 subunits and binds to the IL-12R (IL-12Rβ2 and IL-12Rβ1). STAT 4 signalling results in IFNγ secretion and Th1 development.

A potential role for the IL-23/IL-17 axis in atherosclerosis is a growing area of research. mRNA levels of both IL-23 and IL-23R are increased within human carotid plaques with heavy colocalisation to macrophages. Plasma levels of IL-23 are also associated with disease progression and increased mortality (Abbas

et al., 2015). Recently a mouse IL-23R KO model suggest a potential beneficial role for IL-23 in atherosclerosis, suggesting IL-23 regulates foam cell formation (Fatkhullina et al., 2016).

However, a potential role of IL-17 in the pathogenesis of atherosclerosis is still heavily debated. Paradoxical findings have both been reported, with IL-17 showing both protective and exacerbating functions. A detailed review of the findings can be found here (Gong et al., 2015). Several publications have as a result given warnings about the use of IL-17 inhibitors and their potential to aggravate atherosclerosis (Gong et al., 2015, Taleb and Tedgui, 2017).

1.5 IL-36 in psoriasis

Interleukin-36 (IL-36) cytokines are part of the wider IL-1 family and include IL-36α, IL-36β, IL-36γ and their inhibitor, the IL-36 receptor antagonist (IL-36Ra) (Dinarello et al., 2010). Both IL-36α and IL-36γ have been implicated in psoriasis pathology, but IL-36γ levels (lesion staining and circulating levels) positively correlate with psoriasis disease severity to a greater degree than IL-36α (D'Erme et al., 2015, Boutet et al., 2016).

Like other IL-1 family members, IL-36γ requires post translational processing. Skin equivalent models have shown that this is achieved by the protease cathepsin S, which is a serine protease with a role in antigen presentation (Ainscough et al., 2017). The other IL-36 family cytokines are processed by a range of neutrophil proteases such as cathepsin G and neutrophil elastase (Henry et al., 2016).

IL-36 binds to the IL-36 receptor (IL-36R) which is expressed by keratinocytes, fibroblasts and has also been reported on immune cells (Dietrich et al., 2016). Receptor binding is followed by recruitment of an accessory protein (IL-RAcP), which is shared by other IL-1 family members (Towne et al., 2004). Downstream intracellular signalling is poorly characterised, compared to other cytokines but results in MAP kinase and NF-κB activation and the expression of proinflammatory mediators in susceptible cells (Figure 8). Whilst the receptor antagonist is able to bind to the receptor, it does not recruit the AcP, so signalling does not occur and thus the Ra exerts antagonist effects (Towne et al., 2011, Gunther and Sundberg, 2014). IL-36γ is thought to be mainly produced by keratinocytes and other epithelial cells in response to stimuli such as fungi, inflammatory mediators such as TNFα , IL-1, IL-36, bacteria, rhinovirus infection and smoke (Carrier et al., 2011, Chustz et al., 2011, Bochkov et al., 2010, Vos et al., 2005, Parsanejad et al., 2008). IL-36 members have stimulatory effects on a range of cell types including epithelial cells, fibroblasts and immune cells (Chustz et al., 2011, Foster et al., 2014, Mutamba et al., 2012, Johnston et al., 2011). IL-36 expression by epithelial cells has been documented in several tissues including the lung and gut; however, the majority of research has focused on the skin and specifically psoriasis (Chustz et al., 2011).

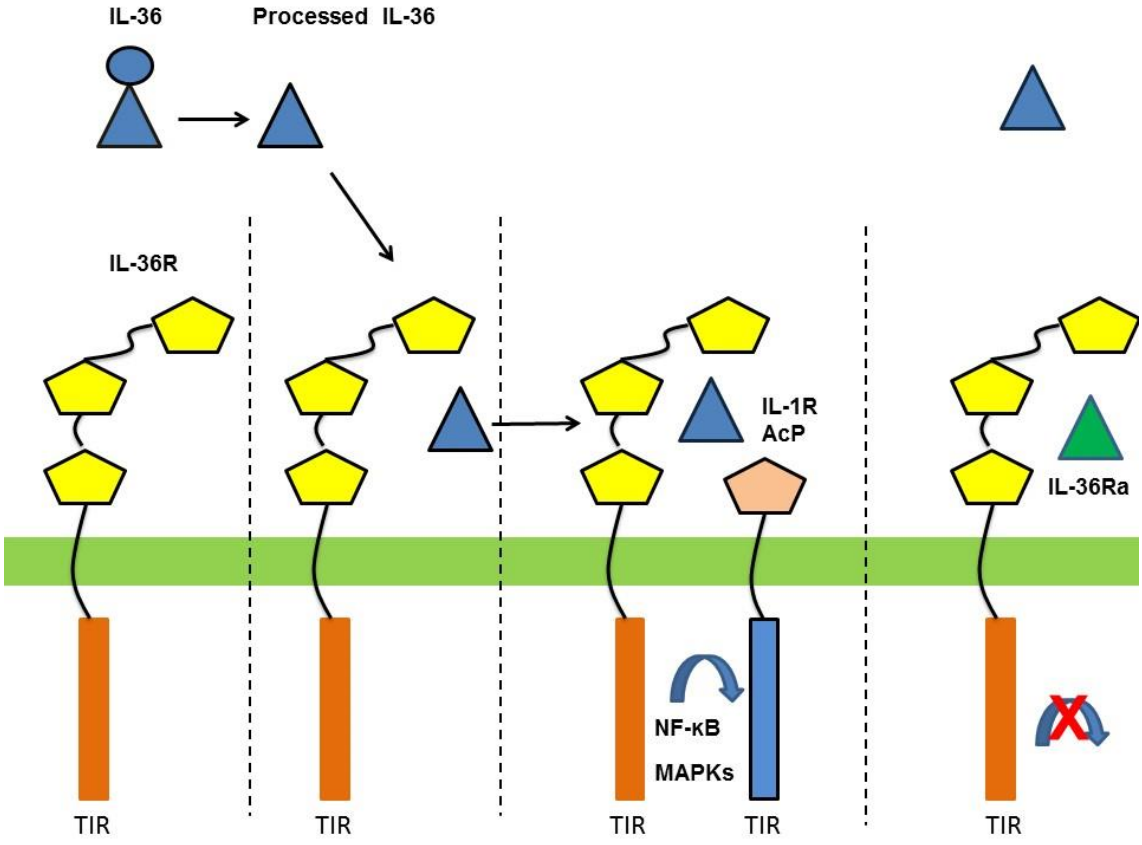


Figure 9: IL-36 and IL-36R

Processed and fully active IL-36 binds to the IL-36R. When the AcP is recruited this leads the TIR domains of the two receptor chains to engage and induce transcription factors such as NF-κB and MAPKs. The IL-36Ra however does not recruit the AcP, so does not result in signalling. TIR: Toll/interleukin-1 receptor. Adapted from (Gabay and Towne, 2015).

IL-36γ is highly upregulated in psoriasis lesions on both the mRNA and protein level (D'Erme et al., 2015). Expression levels of IL-36γ also correlate with levels of other proinflammatory cytokines such as IL-17, IL-23 and TNFα in psoriasis lesions (Carrier et al., 2011). Transgenic mice in which keratinocytes overexpress IL-36α are susceptible to psoriasis-like inflammation following 12-O-Tetradecanoylphorbol-13-acetate (TPA) treatment (Blumberg et al., 2010). Using the same model, mice which were deficient in the IL-36Ra showed chronic skin

abnormalities and enhanced psoriasis plaque development. Mice deficient in the IL-36R were protected from plaque development (Tortola et al., 2012). Also of note, generalized pustular psoriasis (GPP), a severe form of psoriasis, has been linked to a mutation in the IL-36Ra. This mutation results in a less stable protein with resultant reduced control over IL-36 mediated responses (Marrakchi et al., 2011). The subsequent condition has been termed DITRA (deficiency of the IL-36 receptor antagonist) (Cowen and Goldbach-Mansky, 2012).

A potential pathway of action for IL-36γ in psoriasis has recently been proposed (Gabay and Towne, 2015). IL-36γ is released by keratinocytes in response to environmental factors and further stimulates keratinocytes to release chemokines in an autocrine manner and DCs/Langerhans cells in a paracrine manner. DCs/Langerhans' cells then produce IL-1, IL-6 and IL-23, which can stimulate Th17 responses. Th17 responses lead the secretion of several cytokines important in psoriasis such as IL-22 and the typical manifestations of psoriasis pathology such as DC and neutrophils recruitment.

1.6 The importance of endothelial cells in psoriasis

Psoriatic plaques are caused by an imbalance of proinflammatory mediators, which attract leukocytes and stimulate proliferation of keratinocytes. Vascular changes within psoriasis lesions have been gaining attention for many years. Capillaries at lesion sites are often dilated and tortuous in arrangement (Bull et al., 1992).

1.7 Angiogenesis in psoriasis

Angiogenesis is the formation of new blood vessels from the pre-existing vasculature and is a hallmark of psoriasis lesions (Heidenreich et al., 2009). Microvascular changes within psoriasis lesions include pronounced dilation, increased permeability and endothelial cell proliferation. Immature permeable blood vessels may enhance dermal inflammation through immune cell recruitment (Braverman and Sibley, 1982, Braverman and Yen, 1977). A recent study confirmed a positive correlation between hypervascularization and disease severity in psoriasis patients (Rosina et al., 2009). Excessive capillary-venular dilatation precedes development of psoriatic inflammation, and resolution of these vascular changes is associated with remission of psoriasis lesions (Kulka, 1964). VEGF-A is thought to be the driving force behind angiogenesis observed in psoriatic lesions. Mice which overexpress VEGF-A show an inflammatory response that histologically resembles psoriasis (Detmar et al., 1998, Xia et al., 2003). The VEGF-A gene is located on chromosome 6 at 6p21, close to PSORS 1, which is a known chromosomal locus for psoriasis susceptibility (Trembath et al., 1997, Brogan et al., 1999). The +405 CC VEGF genotype, also known as the "high VEGF producing genotype" is associated with early onset psoriasis, whereas the "low VEGF producing genotype" has no association with psoriasis (Diaz et al., 2000, Young et al., 2006, Detmar, 2004). This suggests that the pro-angiogenic potential of an individual may influence disease progression.

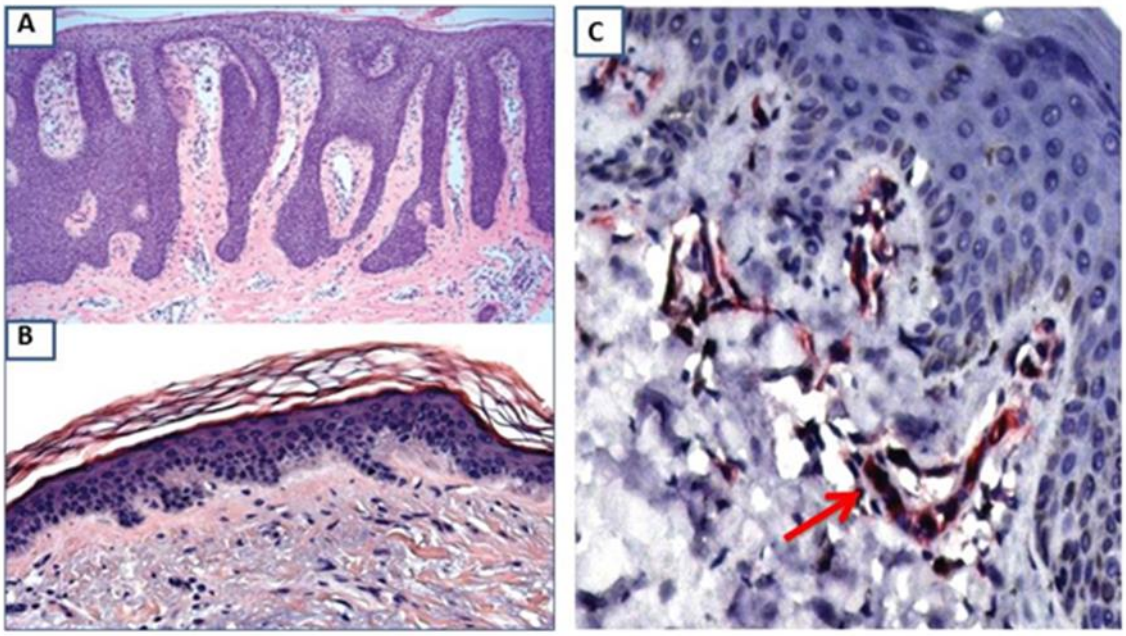


Figure 10: Histological stains of Psoriasis

***A) Psoriasis lesion showing epidermal thickening. B) Healthy skin showing normal epidermis. C) Blood vessels stained in red in a psoriasis lesion*.**

Images taken from (Heidenreich et al., 2009).

1.8 How do endothelial cells contribute to inflammation in the skin?

Inflammation can be either acute or chronic, as seen in psoriasis. Acute inflammation usually revolves in a few days. Vascular dilation and permeability is seen in acute inflammation and is associated with the activation and recruitment of neutrophils (Wright et al., 2010, Majno, 1964). Chronic inflammation is associated with a sustained period of leukocyte recruitment and associated with angiogenesis (Jackson et al., 1997). The cellular infiltrate is also different, consisting of macrophages, monocytes and lymphocytes.

As previously described in section 1.1, ECs are central players in inflammation because of their ability to recruit leukocytes to the site of injury. ECs express adhesion molecules ICAM-1, VCAM-1 and E-selection, all of which encourage leukocyte migration to the site of inflammation (Osborn, 1990).

Several currently used medications for psoriasis may also work by affecting ECs in addition to immune cells. Methotrexate is thought to inhibit adhesion molecule expression (Yamasaki et al., 2003). Moreover, Efalizumab, which was efficient for psoriasis treatment when available, (discontinued to adverse side effects) works by blocking LFA-1 on leukocytes so they are unable bind to the adhesion molecule ICAM-1 on ECs (Lebwohl et al., 2003). A number of proinflammatory cytokines upregulated in psoriasis lesions including TNFα, IL-1 and IL-17 are capable of activating ECs (Terajima et al., 1998, Cannizzaro et al., 2014). A detailed description of psoriasis implicated cytokines with roles in endothelial activation can be found in psoriasis systemic section (1.10.1).

1.9 Pro Angiogenic factors in psoriasis

1.9.1 VEGF

Vascular endothelial growth factor (VEGF) is a family of growth factors responsible for new vascular angiogenesis (Keck et al., 1989). Many isoforms of VEGF exist due to mRNA splicing (Nowak et al., 2008). Two distinct families have emerged: the pro-angiogenic (VEGF-Axxx) and anti-angiogenic (VEGF-Axxb) (Nowak et al., 2008).

VEGF binds to the VEGF tyrosine kinase receptor on ECs leading to signal transduction and resulting in proliferation. VEGF can be secreted by a range of cell types including keratinocytes (Bae et al., 2015).

Psoriasis skin, both lesional and uninvolved, contains a higher concentration of VEGF when compared to skin of healthy individuals (Henno et al., 2009). Importantly though, this VEGF is the isoform 121 (VEGF-A121). Whilst this isoform is not thought to be more angiogenic, it does however cause greater vessel dilation and leakage (Kusters et al., 2003) compared to other pro-angiogenic isoforms. This allows greater infiltration of inflammatory cells to the site.

In psoriasis, VEGF is primarily secreted by keratinocytes (Zhang et al., 2005). The reasons why keratinocytes from psoriasis patients secrete increased VEGF levels compared to those from healthy skin are not known. It could be due to genetic predisposition or as a result of stimulation from proinflammatory cytokines (Wongpiyabovorn et al., 2008). Mouse models where keratinocytes over express VEGF result in an enhanced vascular network and a skin condition similar to psoriasis (Detmar et al., 1998). Recently anti-VEGF treatments have also been shown to reduce psoriasis severity (Li et al., 2014). These findings highlight the key role of the endothelium via the importance of angiogenesis in the pathology of psoriasis as well as highlighting the role of VEGF in worsening disease (Keshtgarpour and Dudek, 2007, Akman et al., 2009).

The involvement of VEGF could provide a possible link to atherosclerosis. Angiogenesis and new vessel growth is found in atherosclerotic plaques and VEGF plays a similar role in promoting vessel permeability, thus enhancing leukocyte recruitment and inflammation, most importantly macrophages (Celletti et al., 2001). Indeed, angiogenesis is associated with plaque instability and rupture in atherosclerosis (Zhang et al., 1993). Interestingly higher VEGF serum levels are associated with psoriasis severity and can be used to predict adverse

cardiac events (Heeschen et al., 2003). The VEGF121 isoform could possibly have systemic effects and cause the atherosclerotic plaque vessels to be more 'leaky' and thus inflamed and more prone to rupture.

1.9.2 Angiopoietins in psoriasis

The Angiopoietin growth factors Ang-1 and Ang-2 and their tyrosine kinase receptor Tie2 play a role in angiogenesis. The Ang-Tie2 pathway is involved in maturation, stabilization and growth of new vessels (Maisonpierre et al., 1997). The Ang1-Tie2 pathway leads to vessel stabilization and maintenance during vascular embryogenesis (Sato et al., 1995). The Ang2-Tie2 pathway causes vessel destabilization, meaning the vessels begin to sense for growth and survival signals, and when pro-angiogenic factors are present, this leads to angiogenesis (Maisonpierre et al., 1997). Ang1 can be produced by a range of cells, but Ang2 seems to be limited to ECs (Kuroda et al., 2001).

The Ang/Tie2 pathway is activated during psoriasis, predominately in the papillary dermis. Transgenic mouse models in which ECs and keratinocytes over express Tie2 results in a disease similar in appearance and pathology to psoriasis and reversal of Tie2 expression reduced the disease (Voskas et al., 2005). There is also evidence that Ang-2 may sensitize ECs to proinflammatory cytokines such as TNFα by influencing adhesion molecule expression (Fiedler et al., 2006).

Whilst it is known the Ang/Tie2 pathway is upregulated in psoriasis lesions, no study has considered if ECs from uninvolved skin show different expression levels of proteins of this pathway compared to healthy patient ECs. A potential genetic upregulation of this pathway would increase angiogenesis and the likelihood of plaque rupture in atherosclerosis. Recently, a single nucleotide

polymorphism (SNP) of Ang2 has been associated with psoriasis patients (He et al., 2015). However, no functional significance has been attributed to this SNP. It should also be noted that Ang2 levels have been shown to be reduced following successful psoriasis treatment (Kuroda et al., 2001). These findings hint at an important role of Ang2 in psoriasis disease maintenance.

1.10 Do ECs from psoriasis patients have a genetic predisposition for atherosclerosis development?

There is some evidence that ECs from psoriasis patients may have certain genetic predispositions which could aid atherosclerotic development. These are discussed below.

1.10.1 Genetics

There are many known immune-genetic pre-dispositions that are associated with psoriasis. These genes have many wide-ranging functions including antigen presentation, T cell development/polarisation and innate immunity. An excellent review of the immunogenetics of psoriasis can be found here (Harden et al., 2015).

1.10.2 NF-κB

NF-κB is a transcription factor which has a key role in immune responses in the majority of cell types (Hoesel and Schmid, 2013). Many different genes that encode components of the NF-κB pathway have been found to be associated with psoriasis and importantly some of these are known to exist in ECs (Goldminz et al., 2013). Under basal conditions, NF-κB is bound to its inhibitor, IκB within the cytoplasm (Perkins, 2007). Upon activation, IκB is phosphorylated by IκB

kinase (IKK) targeting IκB for degradation and NF-κB is released for translocation to the nucleus and as a result, inflammatory genes are expressed (Perkins, 2007). A variety of cellular receptors involved in immune signalling can result in NF-κB activation. In the context of the endothelium and atherosclerosis, increased NF-κB activation could lead to increased adhesion molecule expression (VCAM-1, ICAM-1, E-selectin) and chemokine secretion, all of which would increase leukocyte adhesion (Osborn, 1990).

1.10.3 CARD 14

CARD (Caspase recruitment domain family member) proteins are scaffolding proteins involved in the activation of NF-κB (Blonska and Lin, 2011). Related CARD family members interact with molecules involved in the recruitment and activation of IKK, such as MALT1 and TRAF2 (Scudiero et al., 2011, Bertin et al., 2001). A coding SNP in CARD14 was found to be associated with psoriasis in genome wide studies (Jordan et al., 2012a). The psoriasis mutation associated with CARD14 results in increased NF-κB activation (Jordan et al., 2012b, Harden et al., 2014). Psoriasis patient dermal ECs were found to express CARD14 and have increased NF-κB activation which resulted in increased chemokine production. After transfection of psoriasis CARD14 into healthy dermal ECs, increased NF-κB activation occurred (Harden et al., 2014). CARD14 is known to be expressed in all ECs, so it is plausible that the psoriasis mutation is expressed in aortic ECs, which could lead to increased NF-κB activation and accelerated atherosclerotic plaque development.

1.10.4 ZC3H12C

ZC3H12C (zinc finger DHHC-type containing 23) has been shown to significantly dampen EC inflammatory responses. Both overexpression and knock down studies have confirmed that ZC3H12C has the ability to supress NF-κB activation (Liu et al., 2013). However, little is known about the regulatory abilities of ZC3H12C or the exact method of NF-κB suppression. Interestingly, ZC3H12C has a genetic association with psoriasis (Tsoi et al., 2012). However, no functional role has been attributed yet to this gene. It could be possible that psoriasis patients may have a loss of function in ZC3H12C and lose its anti-inflammatory ability, thus allowing enhanced NF-κB activation.

1.11 Do the systemic effects of psoriasis contribute to atherosclerosis?

The systemic effects of psoriasis could be causing enhanced risk of atherosclerosis. There has been much debate considering whether or not psoriasis is a systemic disease. However, multiple studies have found correlation between PASI score and CVD risk (Boehncke et al., 2007). Also of note is that several markers implicated in systemic disease are higher in severe psoriasis patient serum such as C-reactive protein (Coimbra et al., 2010), VEGF (Detmar et al., 1994), P-selectin (Garbaraviciene et al., 2010), and previously mentioned cytokines (Roussaki-Schulze et al., 2005).

Many ‘traditional’ cytokines such as TNFα and IL-1 have well defined roles in stimulating ECs and upregulating adhesion molecules and chemokine secretion

(Aziz and Wakefield, 1996). TNFα inhibitors are well established treatments for psoriasis (Table 2) and a general consensus suggests they may also potentially lower CVD risk (Yang et al., 2016). However, recently a number of 'newer' cytokines have been described as also possessing the ability to activate the endothelium (Table 1).

CYTOKINE	+ OR - IN PSORIASIS SERUM	REPORTED ENDOTHELIAL FUNCTIONS	Reference
IL-10	-	Anti-inflammatory, downregulates chemokines and adhesion molecules	Jacob et al., 2003,Krakauer, 1995, Lisinski and Furie, 2002, Potteaux et al., 2006
IL-17	+	Upregulates adhesion molecules and chemokine secretion	de Oliveira et al., 2015, Michalak-Stoma et al., 2013,Xing et al., 2013
IL-19	Both + and - reported	Reduces adhesion molecule mRNA stability- thus reducing leukocyte adhesion. Also promotes angiogenesis	Li et al., 2005, Witte et al., 2014,England et al., 2013
IL-22	+	Promotes angiogenesis	de Oliveira et al., 2015, Shang et al., 2015
IL-22BP	Unknown	Inhibitory binding protein to IL-22	Martin et al., 2014
IL-23	+	No described effects, however the IL-23p19 chain has the ability to upregulate adhesion molecules	Michalak-Stoma et al., 2013, Espigol-Frigole et al., 2016
IL-33	+	Upregulates adhesion molecules alone and synergistically with TNF-α	Baliwag et al., 2015, Hueber et al., 2010, Mitsui et al., 2016, Pollheimer et al., 2013, Choi et al., 2012
IL-36γ	+	Adhesion molecule upregulation and chemokine secretion	Bridgewood et al, 2017
IL-36Ra	Unknown	Antagonist to IL-36	
IL-37	Unknown	Anti-inflammatory- downregulates adhesion molecules, but promotes angiogenesis	Li et al., 2011

Table 1: Cytokines measured in psoriasis serum, either elevated or decreased, and their known actions on the endothelium

IL-33

IL-33 is an IL-1 family member cytokine produced by fibroblasts, epithelial cells and immune cells (Schmitz et al., 2005, Akdis et al., 2016). IL-33 binds to a receptor complex of Interleukin 1 receptor-like 1 (IL1RL1) and IL-1 Receptor Accessory Protein (IL1RAP) and activates NF-κB in susceptible cells (Chackerian et al., 2007, Pollheimer et al., 2013). Contrasting studies have reported it to be both upregulated and down regulated within psoriasis lesions, however it has been reported as being increased in psoriasis serum (Baliwag et al., 2015, Hueber et al., 2010, Mitsui et al., 2016). Little is known about its role in psoriasis but it could be involved in keratinocyte activation (Balato et al., 2012). Despite recent studies showing IL-33 can upregulate adhesion molecules alone or in synergy with TNFα (Pollheimer et al., 2013, Choi et al., 2012), mouse models of atherosclerosis have produced surprising findings. Systemically administered IL-33 surprisingly appeared to be atheroprotective to mice, whereas endogenously produced IL-33 was shown to have no effect on atherosclerosis severity (Miller et al., 2008, Martin et al., 2015).

IL-17

While IL-17 is mainly produced by T helper 17 cells (Th17 cells) other T cells are also capable of secreting it, such as CD8 T cells (Takatori et al., 2009, Miossec, 2009)**.** When bound to the IL-17 receptor (IL-17R), IL-17 induces the expression of inflammatory mediators in target cells such as keratinocytes and fibroblasts through NF-κB activation (Shen et al., 2006). IL-17 family members A, F and C have all been reported as being over expressed within psoriasis lesions (Harper

et al., 2009, Johansen et al., 2009, Lowes et al., 2008). Elevated levels of IL-17A in psoriasis serum have been reported (de Oliveira et al., 2015, Michalak-Stoma et al., 2013). IL-17A has been shown to induce inflammatory effects on ECs such as adhesion molecule and chemokine expression (Xing et al., 2013). However, various atherosclerosis studies and models of IL-17 have produced paradoxical findings, with both pro-atherogenic and atheroprotective results, a review can be found here (Taleb et al., 2015). The use of IL-17 inhibitors such as Secukinumab for psoriasis have thus far shown no change in CVD risk (van de Kerkhof et al., 2016).

IL-22

IL-22 is a IL-10 family cytokine which is secreted by Th17 and T helper 22 cells (Th22 cells) (Sonnenberg et al., 2011). IL-22 signals via a class 2 cytokine receptor (IL-22R) and activates STAT3 (Signal transducer and activator of transcription 3) (Radaeva et al., 2004, Nagalakshmi et al., 2004). IL-22 is thought to be important in tissue repair and proliferation, however many studies have shown that dysregulated IL-22 action can promote pathological inflammation and tissue destruction, a theory linked to its potential role in psoriasis. A naturally occurring soluble inhibitor, the IL-22 binding protein (IL-22BP) is thought to act as a endogenous regulator preventing the exaggerated effects of IL-22 and has shown to be secreted by dendritic cells (Martin et al., 2014). However, recent research in liver fibrosis showed contradictory, with the IL-22BP aggravating fibrosis and IL-22 protecting (Sertorio et al., 2015). The paradoxical effects of IL-22 and the balance between IL-22 and the IL-22BP in disease requires further research. The IL-22R is known to exist on a variety of cells including ECs with. IL-22 promotes proliferation and angiogenesis of EC (Shang et al., 2015). With

the discovery of the natural inhibitor of IL-22, IL-22BP, it is possible ECs also may also secrete this inhibitor. IL-22 psoriasis serum levels have been reported to be higher when compared to healthy controls (de Oliveira et al., 2015). A monoclonal antibody against IL-22 (Fezakinumab) was undergoing trials for psoriasis, but was discontinued (Gudjonsson et al., 2012).

IL-23

IL-23 is a heterodimeric cytokine and consists of the IL-23p19 subunit paired with the IL-12p40 subunit, the latter being shared with IL-12. IL-23 signals through a receptor complex consisting of the IL-23 receptor (IL-23R) and IL-12Rβ1 (also shared with IL-12) (Duvallet et al., 2011). IL-23 is secreted by inflammatory myeloid cells and plays a central role in Th17 responses. The IL-23/IL-17 axis has been implicated heavily in a range of inflammatory diseases including psoriasis (Iwakura and Ishigame, 2006). IL-23 has also been reported as being elevated within psoriasis serum (Michalak-Stoma et al., 2013). Whilst no effects of IL-23 on ECs have been observed, it has recently been reported that ECs produce intracellular IL-23p19 and that it can influence VCAM-1 expression (Espigol-Frigole et al., 2016). mRNA levels of both IL-23 and IL-23R are increased within human carotid plaques and plasma levels of IL-23 are also associated with disease progression and increased mortality (Abbas et al., 2015). Mouse models with knockout IL-23 or IL-23R have not been explored in relation to atherosclerosis. However, briakinumab, a monoclonal antibody against the P40 subunit of IL-12 and IL-23 was terminated mid trial for psoriasis treatment due to concerns linking it with major adverse cardiovascular events (Traczewski and Rudnicka, 2012). Another agent, Ustekinumab which targets the same

subunits, has been reported as having both no effect or potential reduction in CVD risk (Hugh et al., 2014).

Anti-inflammatory cytokines, IL-10, IL-19 & IL-37

IL-19

IL-19 is another IL-10 family member cytokine secreted by both immune cells and epithelial cells. The exact role of IL-19 in psoriasis is unclear, but it has recently been shown to be the most upregulated cytokine in psoriasis lesions (Baliwag et al., 2015). It binds to the same receptor complex as another family member, IL-20, and is able to induce STAT3 activation (Dumoutier et al., 2001). It has been shown to be produced by keratinocytes and promote keratinocyte growth factor in an autocrine manner. Its concentration has been reported as being both lower and higher in psoriasis serum (Li et al., 2005, Witte et al., 2014). ECs are capable of secreting IL-19 themselves under pro-inflammatory conditions (Jain et al., 2011). IL-19 has shown to be a chemoattractant for ECs and also to promote angiogenesis (Jain et al., 2011). Perhaps most importantly, IL-19 has the ability to interfere with leukocyte-EC adhesion. IL-19 has been shown to lower TNFα induced adhesion molecule-leukocyte interaction. The mechanism remains unclear, as IL-19 does not reduce TNFα induced NF-κB activity but does reduce adhesion molecule mRNA stability (England et al., 2013). Mouse models of atherosclerosis have also produced positive results. IL-19 treated mice show reduced atherosclerosis development, and IL-19 KO mice have exacerbated atherosclerosis development (Ellison et al., 2013, Ray et al., 2015).

IL-10

IL-10 is an anti-inflammatory cytokine secreted by a range of immune cells. IL-10 is capable of reducing chemokine and adhesion molecule expression. Upon binding to the IL-10 receptor (IL-10R), IL-10 is able to block NF-κB activity and lower adhesion molecule expression (Krakauer, 1995, Lisinski and Furie, 2002, Potteaux et al., 2006). Consistent with its anti-inflammatory role, mice models in which IL-10 is over-expressed show reduced atherosclerosis development (Han et al., 2010). Its circulating levels have been reported as being lowered in psoriasis patients (Jacob et al., 2003).

IL-37

IL-37 is an anti-inflammatory cytokine and a member of the wider IL-1 family secreted by monocytes. IL-37 employs the IL-18 receptor alpha (IL-18Rα) and IL-1R8 (SIGIRR) in a receptor complex and is able to lower NF-κB activation that has been induced by Toll/interleukin-1 receptor (TIR) binding (Nold-Petry et al., 2015, Nold et al., 2010) in a range of cell types. IL-37 is able to lower NF-κB induced EC adhesion molecule expression (Li et al., 2011). Recent research has shown IL-37 may have a role in psoriasis skin, with studies showing it is down regulated within lesions (Keermann et al., 2015). However, no study has measured its circulating levels in psoriasis. However, the cytokine has been found to be elevated in the serum of other inflammatory diseases such as rheumatoid arthritis (Keermann et al., 2015). The potential protective role of IL-37 in preventing atherosclerosis development is a growing area of research that is starting to gain momentum (Chai et al., 2015, McCurdy et al., 2014, Wu et al.,

2013). A SNP in IL-37 has been shown to confer significantly increased risk for coronary artery disease (Yin et al., 2017).

Interestingly, two cytokines that may have anti-inflammatory effects, IL-10 and IL-19 have been reported as being reduced in psoriasis serum while the serum status of IL-37 is unknown.

BIOLOGIC	TARGET	FDA APPROVAL
Etanercept	TNFα	2004
Infliximab	TNFα	2006
Adalimumab	TNFα	2008
Ustekinumab	P40 subunit of IL-23 and IL-12	2009
Secukinumab	IL-17A	2015
Tildrakizumab	p19 subunit of IL-23	Phase III
BI 655066	p19 subunit of IL-23	Phase II
Guselkumab	p19 subunit of IL-23	Phase III
ABT-122	IL-17A and TNFα	Phase II
Briakinumab	P40 subunit of IL-23 and IL-12	Terminated
Fezakinumab	IL-22	Discontinued after phase I

Table 2: Biologics currently approved or undergoing clinical trials for the treatment of psoriasis

1.12 Chronic Inflammation in psoriasis as a contributor to CVD?

Multiple studies have shown psoriasis and atherosclerosis involve similar T cell responses such as Th1 and Th17 (Armstrong et al., 2011). Th1 cells are recruited to the dermis and activate keratinocytes by secreting IFNy, IL-2 and TNFα (Harden et al., 2014). Both TNFα and IFNy have been shown to initiate and progress atherosclerotic plaques (Branen et al., 2004, McLaren and Ramji, 2009). Th17 cells and their subsequent Th17 cascade are thought to impair the regulation of inflammation and potentially destabilise plaques (Ma et al., 2013). Animal studies have also shown that chronic skin inflammation results in increased aortic inflammation noted particularly for increased T cells and TNFα and IL-17 (Wang et al., 2012). Monocytes are recruited to the skin lesion sites and also have crucial role in atherosclerosis. CCL2/MCP-1 (monocyte chemoattractant protein-1) plays an important role in atherosclerosis by promoting smooth muscle proliferation and also the formation of foamy macrophages (Ziats and Robertson, 1981, Ylä-Herttuala et al., 1991). Elevated MCP-1 levels also correlate with plaque instability and rupture potential (Liu et al., 2012). MCP-1 serum levels are also known to be elevated in psoriasis (Mehta et al., 2013).

A theory known as the ‘Psoriasis March’ hypothesises that the systemic effects of psoriasis may cause insulin resistance which in turn causes endothelial dysfunction (Boehncke et al., 2011). Insulin is a vasoactive hormone that increases vasodilation through the nitric oxide pathway, so thus can be viewed

as atheroprotective. Activation of the pathway results in eNOS phosphorylation, the precursor enzyme for NO synthesis (Kuboki et al., 2000). NO is essential for vascular homeostasis and its depletion is associated with numerous vascular pathological conditions such as hypertension, hypercholesterolemia and complications associated with diabetes mellitus (Hermann et al., 2006, Feron et al., 1999, Honing et al., 1998). During systemic inflammation, proinflammatory cytokines activate stress kinases such as JNK and P38, which then negatively regulate insulin receptor substrate (IRS-1) thus blocking P13-K/Akt signalling (Copps and White, 2012) which normally activates eNOS and leads to production of NO and dilatation as well as suppression of adhesion molecule expression. However, at the same time, the insulin dependent activation of the MAPK pathway remains undisturbed, which in turn results in increased adhesion molecule expression and decreased NO production (Muniyappa et al., 2007, Sitia et al., 2010). Psoriasis derived dermal ECs have recently been shown to have decreased activation of Akt (Schluter et al., 2016). Also of note, a psoriasis relevant 'cytokine cocktail' could prevent Akt phosphorylation under insulin conditions (Schluter et al., 2016).

IRS-1 is a protein involved with insulin receptor signalling, and is linked to NO production in ECs. Insulin resistant patients are often found to have decreased IRS-1 levels (Jansson et al., 2003). With respect to atherosclerosis development, lower vascular IRS-1 levels have also been seen to be a marker for arterial stiffness (Sandqvist et al., 2005). Whilst no study has considered IRS-1 levels in psoriasis, there has been research into endothelial dysfunction in psoriasis. Ultrasound studies all hint that the psoriasis vessels (endothelium and SMC) may

have impaired functions such as dilation (Gisondi et al., 2009, Ulusoy et al., 2010, Karadag et al., 2010).

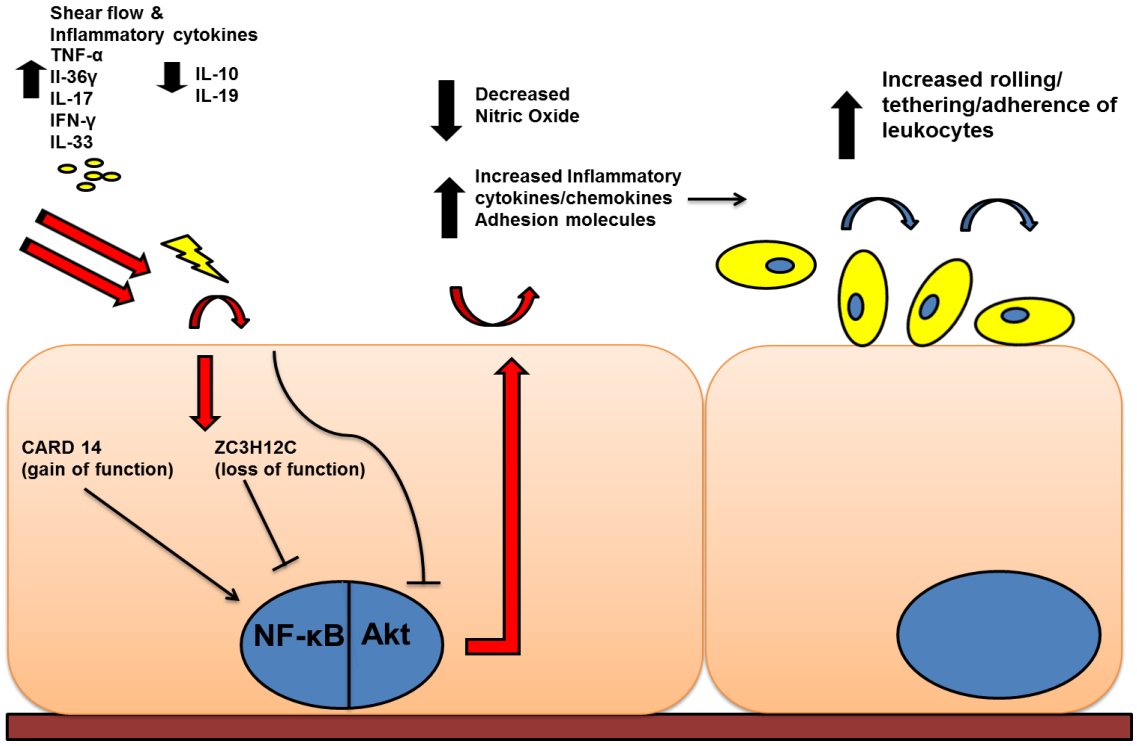


Figure 11: The combined systemic and genetic effects of psoriasis that could enhance atherosclerosis development

Inflammatory cytokines are elevated in psoriasis serum, which leads to increased EC activation. The cytokine induced activity of NF-κB is enhanced by CARD14 mutations. Mutations in ZC3H12C could possibly stop its ability to supress NF-κB, leading to further enhanced NF-κB activation. Anti-inflammatory cytokines such as IL-10 may be lowered in psoriasis serum and able to exert less of a regulatory role. Under normal conditions, insulin binding to ECs would activate Akt leading to nitric oxide production and decreased adhesion molecule expression. Inflammatory cytokines supress insulin-Akt activation, but P38 MAPK activation persists, leading to increased adhesion molecule expression. A combination of

these effects lead to increased rolling tethering and adherence of monocytes on the endothelium and eventually transmigration.

1.12.1 Dyslipidemia

By definition, dyslipidemia is an elevation in cholesterol levels, including LDL-cholesterol, triglyceride or a reduction in HDL cholesterol levels. Dyslipidemia is a well-defined risk factor for atherosclerosis (Koba and Hirano, 2011). Psoriasis' link to dyslipidemia is a growing area of research. Psoriasis patients have an increased concentration of LDL particles and a decrease of HDL particles and also overall decreased particle size for both the aforementioned (Mehta et al., 2012). These abnormalities also show correlation to aortic inflammation when CVD risk factors have been adjusted for (Yu et al., 2012).

1.13 Macrophages in Psoriasis and Atherosclerosis

Numerous comorbidities are associated with psoriasis including cardiovascular disease (CVD). However, one or more clear mechanisms linking psoriasis to CVD are yet to be found. Common theories include endothelial dysfunction, dyslipidaemia and systemic inflammation. Monocytes represent a key cell type in the pathology of atherosclerosis, since they are the main cell type recruited into the intima where they differentiate into macrophages. Through their scavenger receptors, macrophages take up oxLDL and other lipids in a unregulated manner and begin to accumulate in the lesion and form foam cells (Moore et al., 2013).

1.14 Monocyte subsets in inflammation

In recent years, three distinct monocyte populations have been well defined and genotyped depending on their expression of glycoprotein CD14 and FcγIII receptor CD16 (Yang et al., 2014). These subsets consist of the classical (CD14++CD16-) which accounts for 60-70% of total monocytes, the intermediate

monocytes (CD14++CD16+) and the non-classical (CD14+CD16++), which both account for the remaining 10-20% (Golden et al., 2015). Due to their smaller numbers, many studies group intermediate and non-classical monocytes as a single group (CD16+ monocytes) (Rennert et al., 2016).

Several publications suggest that CD16+ monocytes are key mediators of inflammation. An increase in circulating CD16+ monocytes is associated with CVD, acute ischemic heart failure, myocardial infarction and peripheral artery disease (Rogacev et al., 2012, Wrigley et al., 2013). Studies have also recently confirmed that CD16+ monocytes are elevated in psoriasis patients (and correlate with disease severity/PASI), rheumatoid arthritis and inflammatory bowel disease (Golden et al., 2015, Radwan et al., 2016, Koch et al., 2010). The exact mechanism by how CD16+ monocytes are induced or how they contribute to CVD is not clear. CD14+ monocytes appear to be phagocytic while CD16+ appear to be capable of producing enhanced inflammatory mediators (Ziegler-Heitbrock, 2007).

	HEALTHY	PSORIASIS
CLASSICAL	60.6	59.6
INTERMEDIATE	12.0	15.4
NON-CLASSICAL	6.15	6.2

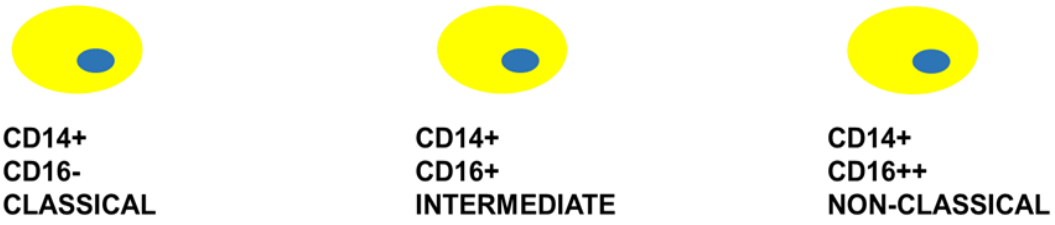


***Figure 12: FACS analysis of monocyte subsets and relative percentage as reported in both healthy and psoriasis peripheral blood by Golden* et al. Note that numbers do not add up to 100% as is primary FACS data.**

Increased CD16+ monocytes in psoriasis have been reported in multiple studies (Golden et al., 2015, Chiu et al., 2010).

1.15 CX3CL1 and monocyte migration

The chemokine CX3CL1 (fractalkine) and its receptor CX3CR1 have been implicated in the process of transendothelial migration of immune cells (Umehara et al., 2001). Uniquely, CX3CL1 exists in two active forms, a membrane bound form which acts as an adhesion molecule, and a soluble form which acts a chemoattractant for immune cells. Endothelial cells upregulate both forms during inflammatory events (Imaizumi et al., 2004). Recently CX3CL1 has gained attention for its preferential role in monocyte subset migration. CX3CR1 is highly expressed by both CD16+ subtypes (intermediate and non-classical) compared to the classical CD14+ (Mandl et al., 2014). While blocking traditional EC adhesion molecules such as E-selectin and VCAM-1 is able to reduce classical

monocyte adherence and migration, blocking of CX3CXL1 is required to stop CD16+ monocyte migration (Rennert et al., 2016). Recently, in an imiquimod induced mouse model of psoriasis, a significant reduction in skin inflammation occurred in CX3CR1 KO mice due to a reduced number of dermal macrophages (Morimura et al., 2016). Previous work has showed psoriasis plaques show heavy infiltration of CD16+ monocytes and also show upregulated dermal CX3CL1 endothelial expression at plaques sites (Golden et al., 2015, Congjun et al., 2015). Numerous studies also correlate serum CX3CL1 positively with PASI score (Congjun et al., 2015).

1.16 CX3CL1-CX3CR1 in CVD

CX3CL1 is recognised as being important in atherosclerosis pathogenesis (Apostolakis and Spandidos, 2013). As well as being a potent monocyte recruiter, it also promotes vascular smooth muscle cell migration, which is a feature of vascular remodelling in CVD (Lucas et al., 2003). Animal studies have shown that blockage of the CX3CL1-CX3CR1 pathway significantly reduces atherosclerosis severity (Teupser et al., 2004). Epidemiology studies have also confirmed a genetically-defined less active CX3CL1-CX3CR1 pathway is associated with a reduced risk of atherosclerosis (Moatti et al., 2001, McDermott et al., 2001, Apostolakis et al., 2009). Whether CX3CL1 has value as a diagnostic marker is still debated, as is also the benefit of its therapeutic inhibition.

1.17 Resident Macrophages in Psoriasis and potential role of IL-36

Mouse models suggest monocytes and macrophages are key players in psoriasis pathology (Clark and Kupper, 2006). Tissue resident macrophages, monocytes and monocyte-derived dendritic cells (MoDC) and their subsequent T cell activating cytokines such as IL-23 have been shown to drive inflammation in imiquimod induced mouse models (Wang et al., 2006, Stratis et al., 2006, Zaba et al., 2009, Fuentes-Duculan et al., 2010). In human psoriasis lesions, monocytes, M1 macrophages and dendritic cell show IL-23p19 staining (Yawalkar et al., 2009). Given that dendritic cells migration is characteristic of chronic lesions, early activation and IL-23 secretion from resident macrophages may represent a key initiation phase. A mechanistic link between IL-36 and the IL-23/IL-17 axis is becoming clear. Mouse models of, psoriasis, respiratory bacterial infection, colitis and renal fibrosis, have all shown IL-36 to be a key component of the axis (Chi et al., 2017, Kovach et al., 2017, Blumberg et al., 2010, Boutet et al., 2016). Numerous autoimmune skin diseases also show a correlation between IL-36 and IL-17 (Zebrowska et al., 2017).

Whilst previous reports have shown IL-36γ induces inflammatory mediators from macrophages, little is known about its ability to induce psoriasis relevant cytokines such as TNFα and IL-23 (Foster et al., 2014). IL-36γ's ability to induce inflammatory mediators from tissue resident macrophages could escalate the inflammatory cascade by activating surrounding fibroblasts, endothelial cells and keratinocytes, and ultimately lead to further immune cell recruitment. Generalised pustular psoriasis patients with DITRA, who received monocyte

apheresis therapy, showed significant improvements in disease severity, highlighting the importance of a potential IL-36-macrophage pathway (Koike et al., 2017, Sugiura et al., 2014).

1.18 IFNγ role in CVD, psoriasis and macrophage activation

Interferons (IFN) are cytokines that mediate anti-viral responses (De Andrea et al., 2002). There are two main classes of interferons. Type I, which include α,β and more. Type I interferons are inducible in a range of cell types and act on macrophages and NK cells to promote anti-viral responses (McNab et al., 2015, Samuel, 1988). Interferon γ (IFNγ) is the only member of the class II interferons and is secreted by activated Th1 cells. Binding to the IFNγ receptor (IFNγR) activates JAK1 and JAK2. As a result STAT1 is phosphorylated (Schroder et al., 2004, Krause et al., 2006, Nathan et al., 1983) and signalling occurs.

IFNγ has a documented importance in atherosclerosis at different stages of the immunopathology and has been described as a master regulator in this disease (McLaren and Ramji, 2009, Gupta et al., 1997). In synergy with TNFα it promotes adhesion molecule expression. IFNγ also promotes SMC proliferation (Zhang et al., 2011, Amento et al., 1991). IFNγ primes and activates macrophages for chemokine/cytokine release and regulates lipid uptake (Li et al., 2010). Th1 cells are thought to drive atherosclerosis development and in mouse models of atherosclerosis, administration of IFNγ increases atherosclerotic lesion size (Whitman et al., 2000). IFNγ is elevated in psoriasis serum and correlates with PASI, giving a potential mechanistic risk for CVD (Abdallah et al., 2009).

2) Effect of IL-36 on endothelial cells

Psoriasis is an immune mediated inflammatory disease affecting 2-3% of the world population (Nestle et al., 2009) and ranges from 1-5% in most developed countires (World Health Organization) IL-36γ is a recently discovered pro-inflammatory cytokine which is highly upregulated in psoriasis lesions on both the mRNA and protein levels (D'Erme et al., 2015). As an IL-1 family member, it shares signalling pathways with IL-1 but the intracellular signalling cascade is poorly described. Expression levels of IL-36γ correlates with levels of other cytokines such as IL-17, IL-23 and TNFα in psoriasis lesions (Carrier et al., 2011) and it is elevated in serum of psoriasis patients (D'Erme et al., 2015). Recently IL-36γ serum levels have been suggested as a biomarker for disease severity due to its correlation with PASI scores (D'Erme et al., 2015).

The pathophysiologic contribution of ECs in psoriatic inflammation is well recognised (section 1.6). The combination of endothelial activation followed by angiogenesis leads to enhanced and sustained leukocyte recruitment/migration to the lesion and thus tissue inflammation which does not resolve (Terajima et al., 1998, Horrocks et al., 1991). Upregulation of adhesion molecules such as VCAM-1 and ICAM-1 have been detected, which recruit T cells and monocytes and leads to extravasation (Cabrijan et al., 2009). However, to date no study has investigated the potential direct effects of IL-36 on EC. Increased serum IL-36γ levels in psoriasis could potentially activate ECs in a systemic manner. EC activation is essential in the early stages of atherogenesis (section 1.1), so confirming a role for IL-36 in EC activation would give strength to a potential mechanism for the psoriasis-CVD risk factor association.

2.1 Hypothesis

I hypothesise that the psoriatic cytokine IL-36, which is thought to be crucial to psoriasis immunopathology, will have pro-inflammatory effects on ECs. This could provide knowledge into the possible enhanced ECs activation and CVD events in psoriasis patients.

2.2 Aims

- Determine if human large and small blood vessel primary ECs in culture respond to IL-36γ by upregulating adhesion molecules (ICAM-1 and VCAM-1) at protein level by FACs analysis
- Determination if the responses of ECs to IL-36 occur through the IL-36 receptor by determining expression of the IL-36 receptor using RT-PCR and ICC and reversal by the IL-36Ra
- Determine which transcription factors IL-36 activates in ECs
- Determine if ECs exposed to IL-36 induce production of IL-8 by ELISA
- Determine if IL-36 stimulated ECs develop chemoattractant ability for T cells
- Determine if IL-36 induces angiogenesis in dermal endothelial cells

2.3 Methods

2.3.1 Ethics

For current and following chapters, blood from both healthy and disease psoriasis patients was obtained by a study that was approved by Yorkshire & the Humber

– Leeds West Research Ethics Committee with written informed consent from all subjects (PDAR study: REC 16/YH/0086).

2.3.2 Cell Culture

All cell culture reagents used were cell culture grade. Endothelial cell growth medium and supplement mix (C-22020) was purchased from PromoCell (Sickingenstrasse, Germany). Penicillin/streptomycin (15140-122), Fungizone® Antimycotic (15290-026) and Trypsin- ethylenediaminetetraacetic acid (EDTA) (0.05%) (25300-054) all obtained from Life Technologies (Paisley, UK). Flasks 75cm^2 with phenolic cap (430725U) were supplied by Corning (New York, USA). Sterile 6 well plates (657160) were purchased from Greiner Bio-One (UK). Disposable sterile pipettes of volume 5, 10 and 25 ml were from Sarstedt (Sarstedtstraße, Germany). Phosphate Buffered Saline (BE17513F) was purchased from Lonza (Verviers, Belgium). Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, H3375), bovine serum albumin (A9576), gelatine (G1393), trypan blue (T6146), paraformaldehyde (P6148), Tween20 (P2287) were all from Sigma-Aldrich Chemical Company (Poole Dorset, UK).

2.3.3 Isolation of HUVEC and HDLEC

HUVEC (Human Umbilical Vein Endothelial Cells) cells were isolated by Ethical Tissue, University of Bradford as described before (Eccles et al., 2008). HDLEC (Human Dermal Lymphatic Endothelial Cells) pooled donor cells were provided by Promocell.

Both HUVEC and HDLEC cells were cultured in PromoCell culture media (C-22010). The media contained 2% v/v foetal calf serum, v/v 0.4% endothelial cell growth supplement, epidermal growth factor 0.1 ng/ml, basic fibroblast growth

factor 1 ng/ml, heparin 90 µg/ml and hydrocortisone 1 µg/ml, Penicillin/streptomycin (100U/100mg/ml) and fungizone (2.5 µg/ml).

2.3.4 Cell counting and viability

Cells in suspension were counted and viability assessed using a haemocytometer. Equal volumes of cell suspension and trypan blue were mixed and the total cells in each of the four corner squares were counted. The total cell count per ml was determined by the following calculation.

(Total cells/4) x 10^4 x 2 (dilution factor)

Viability was calculated on the basis that trypan blue dye causes non-viable cells to absorb the dye. The following calculation was thus used to determine viability.

Viable cell count/ total cell count x 100.

2.3.5 Cryopreserving cells

Following cell counting, cells were centrifuged for 5 mins (1000 xg) and the supernatant discarded. Cell pellet was then resuspended in freezer mix (foetal calf serum, 10% v/v DMSO) at a concentration of 1 x 10^6 cells per ml. 1 ml was then added to cryovials and the vials were stored at -80°C for 24 hr. Following this, vials were transported to liquid nitrogen dewars until further use was required.

2.3.6 Resuscitation of cells

Frozen cells in cryovials were removed from liquid nitrogen and 1 ml of pre-warmed PromoCell media was then added to the cryovial. The suspension was then transferred to 10 ml of media and centrifuged. The supernatant was discarded which removed DMSO. Cell pellet was resuspended in 10ml of media

and transferred to a T75 cell culture flask pre-coated with 10% v/v gelatine solution for 24 hr and rinsed before adding the cells.

2.3.7 Passaging/expansion of cultures

Cells were allowed to grow to confluency in T75 flasks. All flasks were pre- coated with 10% v/v gelatine solution for 24 hr at 4°C and rinsed before addition of cells and media. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Media was changed every 48 hr. Cells were passaged at a ratio of 1:3. Culture media was removed and the cells were washed with PBS. Cells were then treated with trypsin/EDTA (0.05%) at 37°C for 3 mins. The trypsin was then removed without disrupting the cells. The flask was then tapped gently detaching the cells. Cells were then resuspended in required volume of media and aliquoted to new T75 flasks or into plates for experiments.

2.3.8 Immunocharacterisation of ECs

Coverslips were placed in 6 well plates, and both coated with 10% gelatine as described before. ECs were then plated onto the plates in 2 ml media per well. Plates were then incubated 5% CO_2 at 37°C and cells were allowed to reach 90% confluency. Media was then removed and ECs were fixed using 4% w/v formaldehyde solution in PBS for 15 mins at room temperature. Following this, the fixative was removed and cells were washed with PBS. Cells were then blocked with 1% w/v BSA in PBS for 1 hr at room temperature. Cells were then incubated with mouse anti-CD31 (FITC tagged) (Sigma, F8402) or sheep anti-human vWF (both at a dilution of 1 in 20) and incubated overnight at 4°C. As a negative control, relevant FITC isotype controls were used (mouse IgG or sheep IgG) (Abcam, Cambridge, UK). Following this, the cover slips were removed and

counterstained by adding a few drops of 5 –Diamidino-2-phenylindole (DAPI) which also served as mounting media (Sigma, F6057). Cover slips were mounted onto slides and stored in the dark before viewing. The Nikon eclipse 8oi (Nikon DS-U1, Tokyo, Japan) version 5.03 using 20 x objective was used to take images. The FITC channel and DAPI channels were used to visualise CD31 and nuclei respectively.

2.3.9 Generation of active IL-36 proteins

IL-36γ and IL-36Ra were obtained from collaborators from the University of Leeds. Generation methods for the proteins have already been published (Macleod et al., 2016).

2.3.10 Stimulating ECs with IL-36

6 well plates were pre-coated with 10% v/v gelatine solution overnight prior to use. Cells were seeded onto 6 well plates in supplemented media and allowed to reach 90% confluency. Prior to stimulation, media was replaced. Cells were stimulated with various concentrations or combinations of IL-36γ and the receptor antagonist for 24 or 48 hr and incubated as above. Following stimulation, supernatant was removed and stored at -80°C. Cells were harvested for FACS or RNA isolation (see methods below). Experiments were conducted on healthy HUVEC donor cells from 5 different volunteers and a set of pooled donor HDLECs from PromoCell. Relevant cells were incubated with NF-κB inhibitor (IMD- 0354)

(Merck Millipore, Billerica, USA) 1 µmol/L for 1 hr prior to stimulation to test the importance of this signalling pathway.

2.3.11 Measurement of CCL20, CCL2 and IL-8 in supernatant by enzyme-linked immunosorbent assay (ELISA)

CCL2 and IL-8 Ready set ELISA kits (Ready-SET-Go!®- 2nd Generation) were purchased from eBioscience (San Diego, USA). Corning costar 96 well plates (9018) were used for all ELISAs. All assays were conducted at room temperature and kits used as per the manufacturer's instructions. Briefly, for both kits, the capture anti-human antibody was diluted 1 in 250 in coating buffer (1 x PBS). ELISA plate wells were incubated with 100µl capture antibody overnight. Plates were then washed 5 times using 300 µl wash buffer (0.05% tween-20 in PBS). Plates were then blocked with 1 x assay diluent (1% BSA in PBS) for 1 hr. Plates were further washed 3 times with wash buffer. 100 µl sample or standard (recombinant protein) was then added in duplicate. Standard was diluted in 2-fold serial dilution using assay diluent to produce a standard curve. After 2 hr, plates were washed 3 times with wash buffer. Detection antibody (anti-human biotin) was diluted 1 in 250 in assay diluent and 100 µl was added to each well for 1 hr. Plates were then washed 3 times with wash buffer. Enzyme (Avidin-HRP) was diluted 1 in 250 in assay diluent and 100 µl added to each well for 30 mins. Plates were then washed 5 times with wash buffer. 100 µl of substrate (tetramethylbenzidine/TMB) was then added for 15 mins. Reaction was stopped by adding 50 µl stop solution, 1 Molar sulphuric acid, Sigma (35276) to each well.

The absorbance of each well was then measured at 490nm using the Tecan Infinite 200 plate reader using the Magellan 6 analysis software (Tecan, Männedorf, Switzerland).

CCL20 (DY360) ELISA kit was purchased from R&D systems (Minneapolis, USA). Corning costar 96 well plates were used. Capture antibody, mouse anti-human was diluted 1 in 180 in PBS and 100 µl added to each well and left overnight. Plates then washed as above and blocked, and samples/standards added as above. Detection antibody goat anti-human was diluted 1 in 180 in assay diluent and 100 µl added to each well for 2 hr. Assay procedure was then identical to above.

2.3.12 Measurement of adhesion molecules by Fluorescence-activated cell sorting (FACS)

Following cell culture stimulations on 6 well plates, supernatant was removed and stored at -80 °C. Cells were then washed with PBS, harvested using a cell scraper and transferred to tubes. Each tube suspension then had a cell count performed using the previously described method. From this 100,000 cells were transferred from each falcon to a new tube. Cells were then centrifuged for 10 mins (1000 x g) and the supernatant discarded. The cell pellets were then resuspended in 1 ml 5% BSA in PBS for 30 mins to block. Suspension was then centrifuged again as before, the supernatant discarded, and cells were resuspended in 300 µl PBS. The following antibodies (BioLegend, San Diego, USA) were then added, all at 1 in 300 dilution, Alexa Flour 488 anti-human CD54 (ICAM-1) (322713) and PE anti-human CD106 (VCAM-1) (305805). All were raised in mice. Unstimulated control cells were used for single stain, unstained and IgG isotype controls. The isotype controls were Alexa Flour 488 mouse (400132) and PE mouse (400111). After 1 hr incubation, cells were centrifuged as above and resuspended in 300 µl PBS. Cells were then analysed by the Beckman Coulter CyAn™ ADP Analyser, using the Summit software version 4.1.

2.3.13 IL-36 Receptor confirmation on endothelial cells by RT-QPCR

2.3.14 Isolation of RNA

RNA was isolated from EC using Quick-RNA MiniPrep (Zymo Research, Irvine, USA) following manufacturer's instructions. Briefly, following removal of supernatant, cells were washed twice with PBS. 200 µl ZR RNA Buffer was then added to each well and cells lysed through gentle pipetting. The lysate was then transferred into the Zymo-Spin column with a collection tube fitted and centrifuged for 1 min at 12,000 g. The flow-through was then discarded. Following this, 300 µl RNA pre-wash buffer was added to the column and then centrifuged as before and the flow through discarded. 600 µl RNA wash buffer was then added, and the column spun and the flow through discarded as before. The column was then spun as before to ensure complete removal of wash buffer. The column was then placed in an RNase-free tube and 35 µl DNase/RNase free water was added. The column was then centrifuged as before and the eluted RNA was stored at -80°C.

2.3.15 Synthesis of cDNA

Prior to cDNA synthesis, RNA concentration was measured using NanoDrop spectrophotometer and the required amount (1 µg) was used in cDNA synthesis reactions. Isolated RNA was converted to cDNA using RevertAid first strand cDNA synthesis kit (K1662) (Thermo Scientific). 11 µl template RNA was added to a nuclease free tube. The following was then added in order. 1 µl Oligo (dT) primer, 4 µl reaction buffer, 1 µl RiboLock RNase inhibitor (20U/ µl), 2 µl 10mM dNTP mix and 1 µl RevertAid M-MuLV RT (200U/ µl). As a negative control to ensure no DNA contamination when conducting PCR, a tube with no RevertAid M-MuLV RT was included, which should contain no cDNA.

The tubes were then incubated for 5 min at 25 °C and then for 60 min at 42 °C. The cDNA was stored at - 80°C until use.

2.3.16 Receptor confirmation by quantitative PCR (qPCR)

Each reaction well contained the following: 10 µl SYBR green master mix, 6 µl nuclease free water, 2 µl primer (target or housekeeping control) and 2 µl cDNA sample. The following cycling conditions were used: 95°C for 5mins, followed by 40 cycles of 10 seconds at 95 °C and 30 seconds and 60°C. After cycle completion, melt curves were produced to ensure a single product had been amplified. The CFX Connect™ Real-Time PCR Detection System was used for PCR and data analysed using BIO-RAD CFX manager software version 3.0. PCR plates were Bio-Rad (HSP9601). IL-36R expression was assessed using the QuantiTect Primer (QT00072989). U6snRNA (Sigma) was used as a housekeeping gene. The PCR assay was conducted using the Qiagen Quantifast SYBR green PCR kit (204054). Samples were tested in two dilutions and duplicates. A four-point standard curve using HUVEC cDNA was conducted to test primer efficiency. cDNA from the following cell types were used, HUVEC, HDLEC (previously described) and primary keratinocytes and fibroblasts which had previously been isolated and cultured were used as positive controls (Alase et al., 2015). To ensure no DNA contamination was present in the reaction, a water control (instead of sample) was used. To ensure only cDNA had been synthesised, and that results were not from contamination, the no RT control was used.

2.3.17 IL-36 Receptor confirmation by Immunocytochemistry

HUVEC and HDLEC were seeded onto gelatin coated coverslips overnight. Cells were washed in TBS and fixed in 4% formaldehyde for 20 mins. Cells were then blocked for 1 hr in 5% BSA in TBS. Cells were incubated overnight with mouse anti-human CD31 (1:1000) (Dako, Glostrup, Denmark) and rabbit anti-human IL-36R (1:500) (Novus Biologicals, Littleton, USA) or Rabbit IgG isotype control (1:500, Abcam, Cambridge, UK). Cells were washed with TBST and secondary donkey anti-rabbit Alexa 594 conjugated, and donkey anti-mouse Alexa 488 conjugated were added (both 1:1000, both Invitrogen, Carlsbad, USA).NF-kB and c-JUN activation by IHC and western blot.

2.3.18 Chemotaxis Assays on Stimulated EC supernatant

Chemotaxis plates (101-216, 8µm pore size) were purchased from Neuro Probe, USA. 30µl of EC supernatant was placed in the bottom chamber. The porous insert was then placed on top. Blood was obtained from 7 healthy volunteers. Mononuclear cells were isolated from blood by Lymphoprep density gradient centrifugation and resuspended in in RPMI containing 10% FCS. The suspension was then purified using a MACS magnetic separation column (Miltenyi Biotec) using microbeads for CD14 negative selection (to exclude monocytes) followed by CD4 positive selection. T cell purity (>90%) was confirmed by FACS CD4/CD3 selection. Isolated T cells were resuspended in RPMI and 5 x 10^4 cells in 20 µl were added to the top chamber. Cells were incubated in an atmosphere of 95 % humidity and 5% CO2 at 37°C for 2 hr. Following incubation, the suspension in the bottom chamber containing migrated cells was removed and placed in counting slides (BIO RAD, USA, 1450011). The cell count was performed using the BIO RAD TC20 automated cell counter. The percentage of cells that had

migrated was calculated for each treatment. The percentage of cells that migrated for media only was normalised to 0 and other treatment values were expressed as a migration index.

2.3.19 NF-κB and c-JUN analysis by Western blot

Cells were stimulated as before, for 1 hr. Cells were lysed with CelLytic M lysis buffer (Sigma-Aldrich), containing protease an inhibitor cocktail (Roche Applied Bioscience, Rotkreuz, Switzerland) and phosphatase inhibitor (Thermo Scientific, Loughborough, UK). Protein concentration was determined by Bradford Assay, and 20μg of total protein was separated on any kDa mini protean gel (Bio-Rad, Hemel Hempstead, UK). Proteins were blotted onto 0.2 μm PVDF trans-blot pack (Bio-Rad). Membranes were probed with either rabbit anti-human phospho-NF-κB (1:1000), rabbit anti-human Phospho c-JUN (both Cell signalling Technology, Leiden, the Netherlands), or mouse anti-human GAPDH (Santa Cruz Biotechnology, Dallas, USA)(Housekeeping control), in Tris-buffered saline 0.1% Tween-20 (TBST) containing 5% BSA overnight at 4°C. Mouse anti-rabbit or donkey anti-mouse, HRP conjugated secondary antibodies were used at 1:5000 for 1 hr at room temperature. Gels were developed with the addition of ECL substrate (Bio-Rad) and visualised using Bio-Rad chemidoc.

2.3.20 NF-κB and c-JUN phosphorylation by immunocytochemistry

Gelatin-coated coverslips were placed into six-well plates, and HUVEC were cultured and stimulated as before. Following 1 hr of treatment, cells were washed in TBS and fixed in 4% formaldehyde for 20 mins. Cells were then blocked for 1 hr in 5% BSA in TBS. Cells were then incubated overnight at 4°C with either rabbit antihuman phospho-NF-κB (1:1000) or rabbit anti-human phospho-c-JUN

(1:1000) (both Cell Signalling Technology, Leiden, the Netherlands). Cells were washed with TBST, and a secondary donkey anti-rabbit Alexa 594 (1:1000) conjugated antibody was added. Sheep anti-human Von Willebrand factor (FITC conjugated) (Abcam, Cambridge, UK) was used as a background stain.

2.3.21 Angiogenesis assay

Dermal fibroblasts (DF) were provided by the Centre for Skin Sciences, University of Bradford, as described previously (Alase et al., 2015). DF were cultured in DMEM (10% FBS) containing penicillin/streptomycin (100 U/100 mg/mL). DF were allowed to reach confluency in 24 well plates. 6500 HDLEC were then co-cultured with the confluent DF, and then allowed to acclimatise in 1:1 DMEM/ complete Promocell endothelial media overnight. The following day, the media was replaced by endothelial media. The cells were then stimulated with IL-36, IL-36Ra or VEGF inhibitor-sutent (Sigma). Following 5 days, the cells were fixed in 4% formaldehyde for 20 mins and blocked in 5% BSA for 1 hr. Cells were then stained with mouse anti-human CD31 FITC conjugated (Sigma) for 1 hr. Cells were visualised using the EVOS XL microscope (Hatfield, PA, USA).Total tubule length was quantified from each photographic field using the open source software AngioQuant (www.cs.tut.fi/sgn/csb/angioquant) and values averaged.

2.3.22 Statistical analysis

Statistical significance was calculated using a one-way ANOVA with a Bonferroni's multiple comparisons test, unless stated. Analysis was performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Error bars represent the standard error of the mean (SEM). *: $p<0.05$ from indicated controls.

2.4 Results

2.4.1 Immunocharacterisation of EC

For both EC types, the phenotype was confirmed using fluorescence microscopy as described in section 2.2.7. FITC-tagged CD31 (PECAM-1) was used on both cell types and clearly stained all cells.

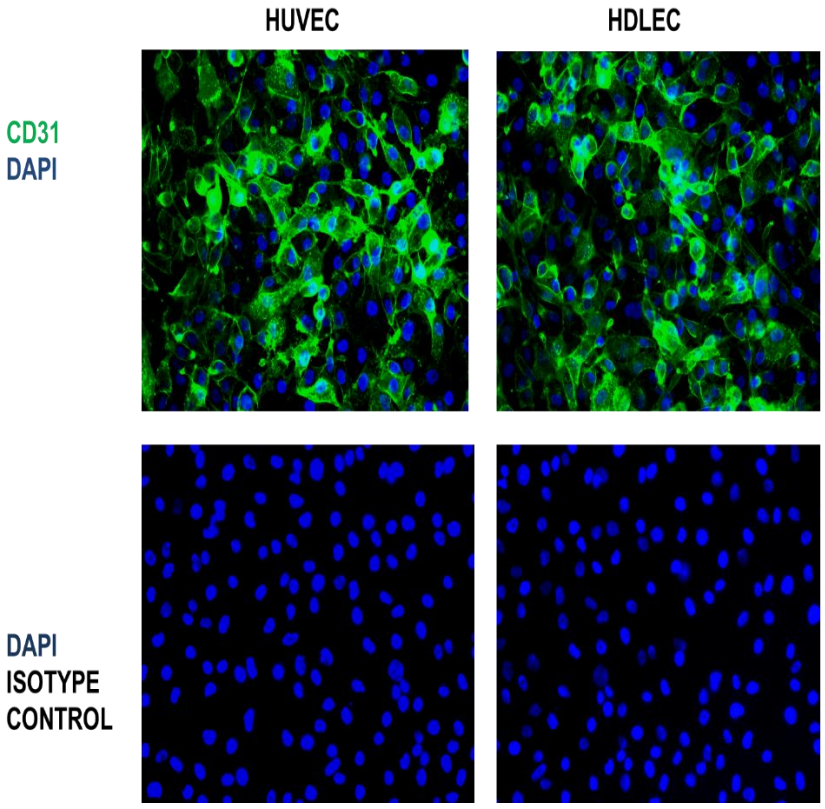


Figure 13: Confirmation of endothelial cell type for both HUVEC and HDLEC

Cells were grown, fixed and then stained with FITC-tagged CD31 and counterstained with DAPI. Isotype control confirms specific binding of conjugated antibody magnification x 40.

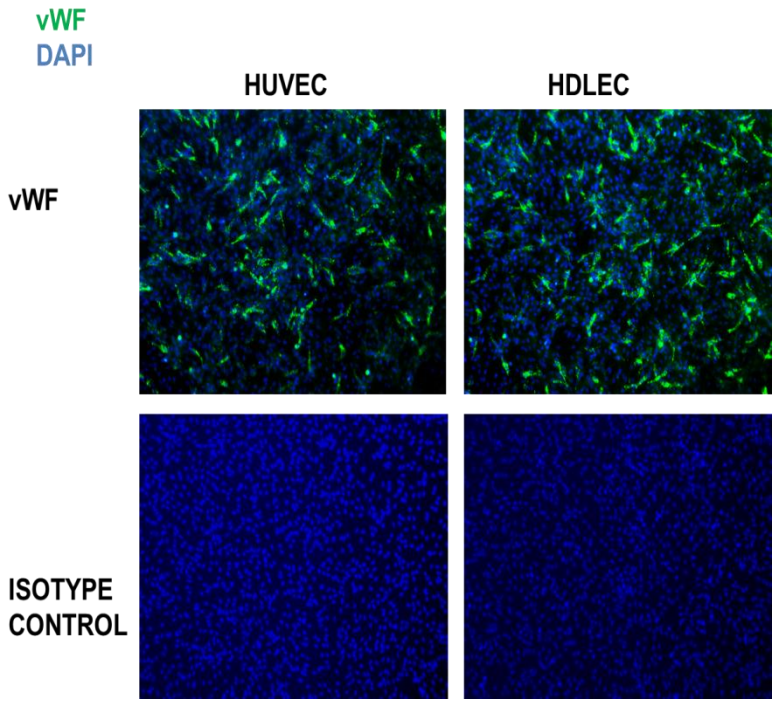


Figure 14: Confirmation of endothelial cell type for both HUVEC and HDLEC

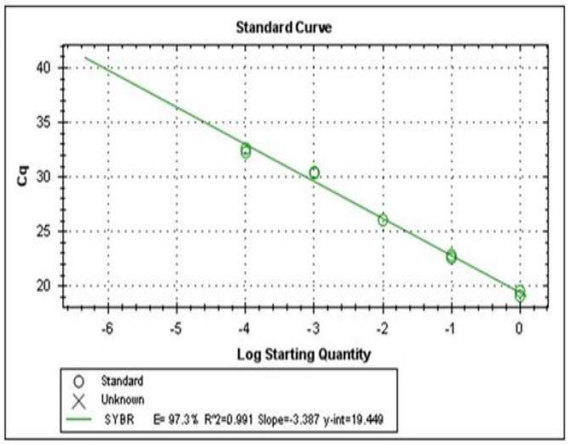
Cells were grown, fixed and then stained with FITC tagged Vwf and counterstained with DAPI. Isotype control confirms specific binding of conjugated antibody magnification x 20.

2.4.2 IL-36R confirmation by RT-QPCR

Whilst no stimulatory effects of IL-36 on ECs have previously been documented, expression of the IL-36R first needed to be confirmed. For analysis of receptor expression, two human EC types were used, HUVEC (Human Umbilical Vein Endothelial Cells) and HDLEC (Human Dermal Lymphatic Endothelial Cells). A range of cell types are known to express the IL-36R including fibroblasts and keratinocytes, and these were used as positive controls. Previous research has shown dermal fibroblasts are highly responsive to IL-36 stimulation (Magne et al., 2006). For both the IL-36R and U6 primers, efficiency curves were generated

(Figure 15). For both primers, suitable efficiency curves were achieved. qPCR analysis confirmed expression of the IL-36R on both HUVEC and HDLEC (Figure 16). Keratinocytes and fibroblasts were used as positive controls.

A) U6



B)IL-36R

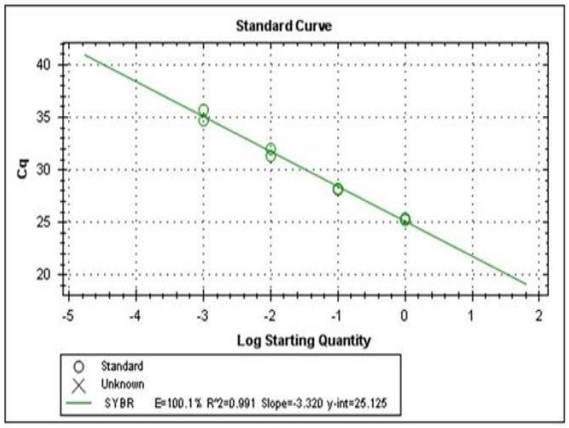


Figure 15 : Efficiency curves for primers U6, housekeeping gene E= 97.3% %, R2= 0.991 . B)IL-36R, E= 100%, R2= 0.991.

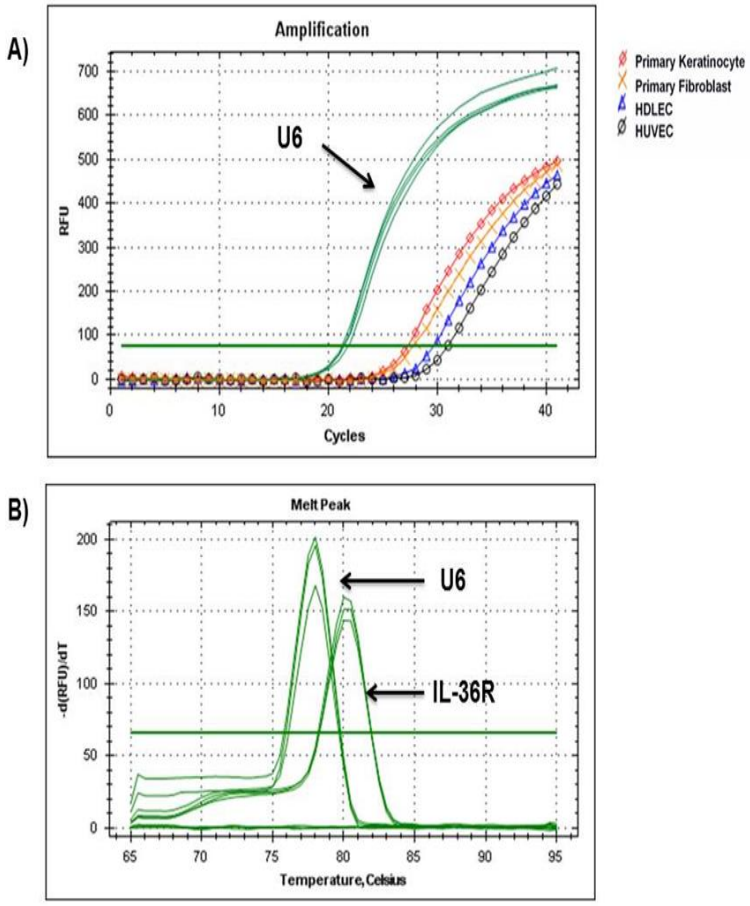


Figure 16: Expression of the IL-36 receptor on cell types

A) Amplification graph showing IL-36R on cell types in comparison to U6 housekeeping gene. B) Melt curves for each cell type cDNA confirm the presence of a single amplified product for both IL-36 and U6.

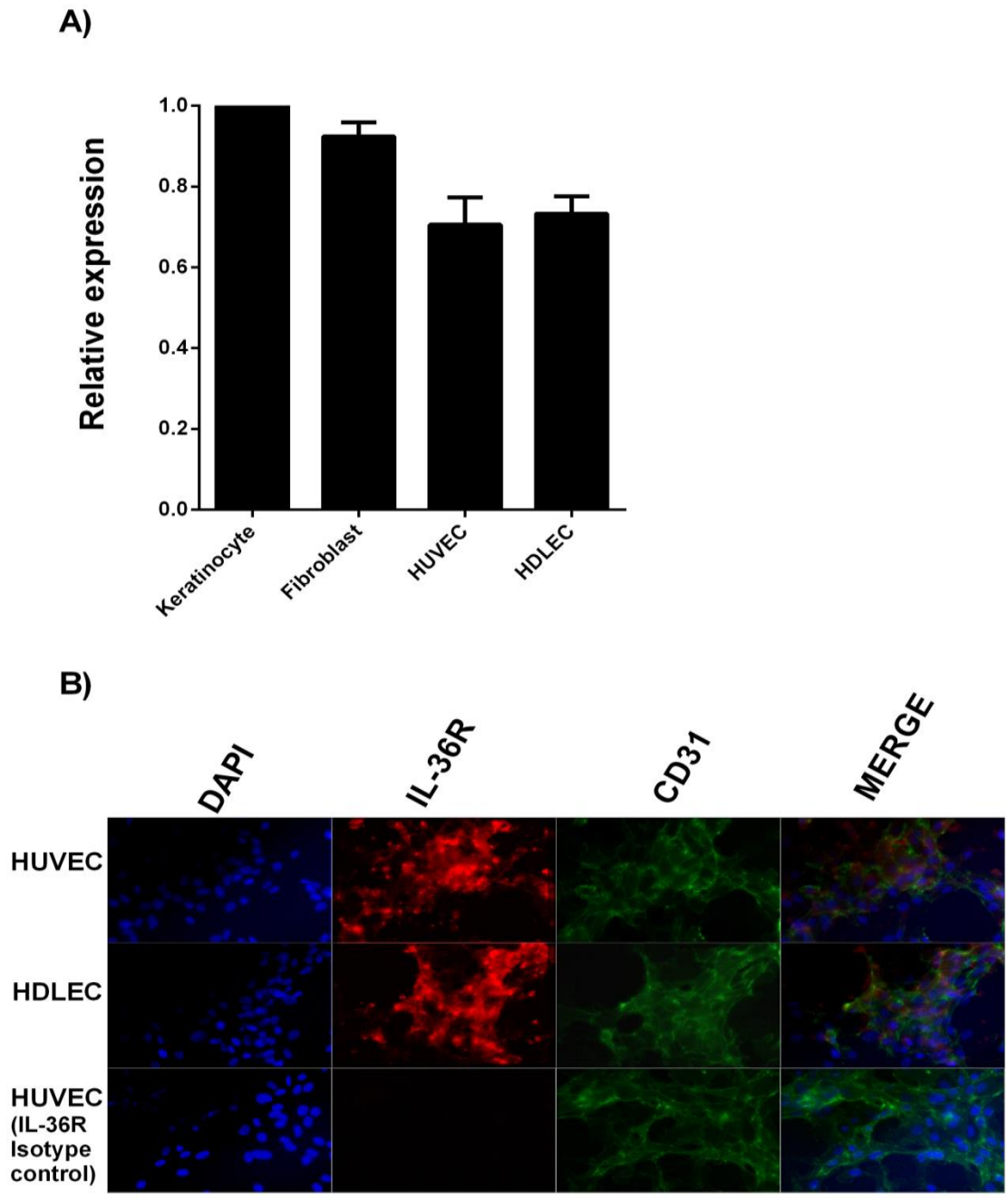


Figure 17: Expression of the IL36R on human endothelial cells

A)qPCR was performed and relative mRNA expression determined. Keratinocyte expression was set to 1.00 and HUVEC, HDLEC and fibroblast expression of the receptor was quantified relative to keratinocyte expression. Samples: HUVEC n = 5, HDLEC n=3 (pooled donors) 3 biological replicates, keratinocytes, fibroblasts n=2. Standard error of mean depicted on graph. B) ICC confirmation of receptor on HUVEC and HDLEC. Magnification x 40.

2.4.3 IL-8 release from ECs following IL-36γ stimulation

To assess possible stimulatory effects of IL-36γ on ECs, IL-8 secretion was measured in response to stimulation. To identify the most effective concentrations and stimulation times for IL-36γ, a range of concentrations and time points were used.

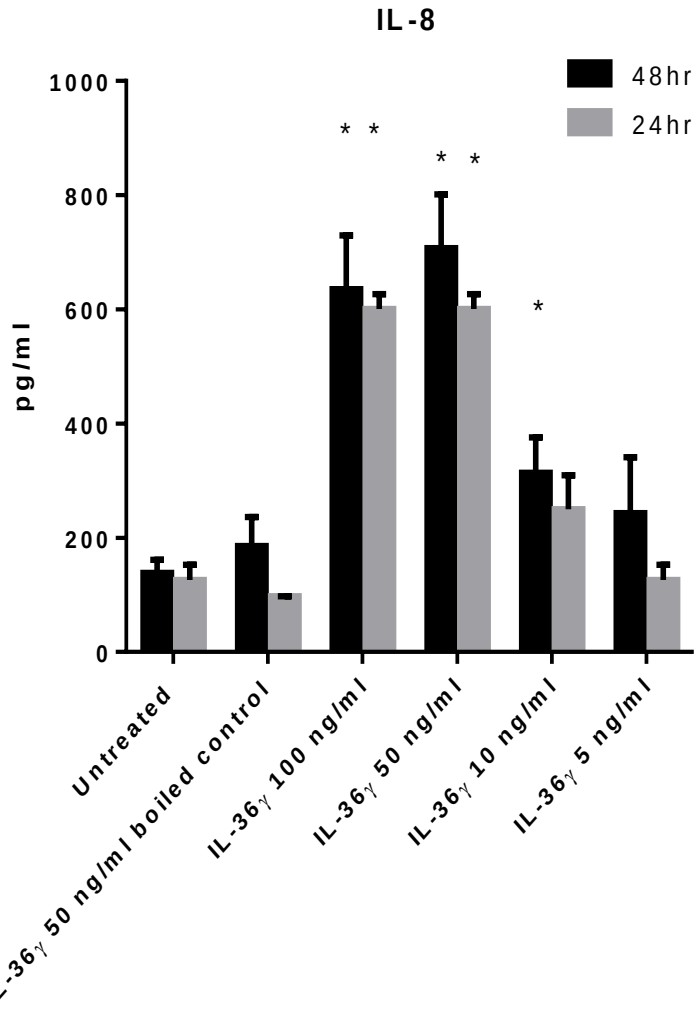


Figure 18: IL-8 secretion by HUVEC following IL-36 stimulation

***IL-8 secretion following IL-36γ stimulation for both 24 and 48 hr stimulations. Sample size, n=4 (average taken of triplicate repetitions per N). ANOVA *: p<0.05 from relevant controls*.**

For both time points a plateau in IL-8 secretion appeared to occur between the concentrations 50 ng/ml and 100 ng/ml. From the statistical significance of the data it was evident the most effective time point was 48 hr and for the concentrations of 10 and 50 ng/ml. No statistical significance was found at 5 ng/ml at either time point and for 10 ng/ml at 24 hr.

2.4.4 Chemokine release from ECs from 48hr IL-36γ stimulation

Following significant IL-8 release following 48 hr simulation with IL-36γ, a range of chemokines known to be secreted by ECs that have pathological significance in psoriasis were measured in duplicate from treated ECs. In addition to IL-8, both types of ECs secreted CCL2 (MCP1) and CCL20 in a dose dependent manner. ECs are known to react very sensitively to endotoxin contamination; the IL-36γ preparation was boiled prior to stimulation to confirm no contamination was present. Boiled controls subsequently produced similar secretion levels to untreated replicates. Both 10 and 50 ng/ml IL-36γ resulted in significant chemokine production when compared to untreated control. The receptor antagonist (IL-36Ra) lowered secretion levels in a dose dependent manner. Increasing concentrations of 10, 50 and 100 ng/ml of the IL-36Ra all lowered IL-36γ mediated secretion of chemokines in comparison to IL-36γ stimulation alone. The effect of IL-36Ra also consolidates the presence of the IL-36R on both EC types.

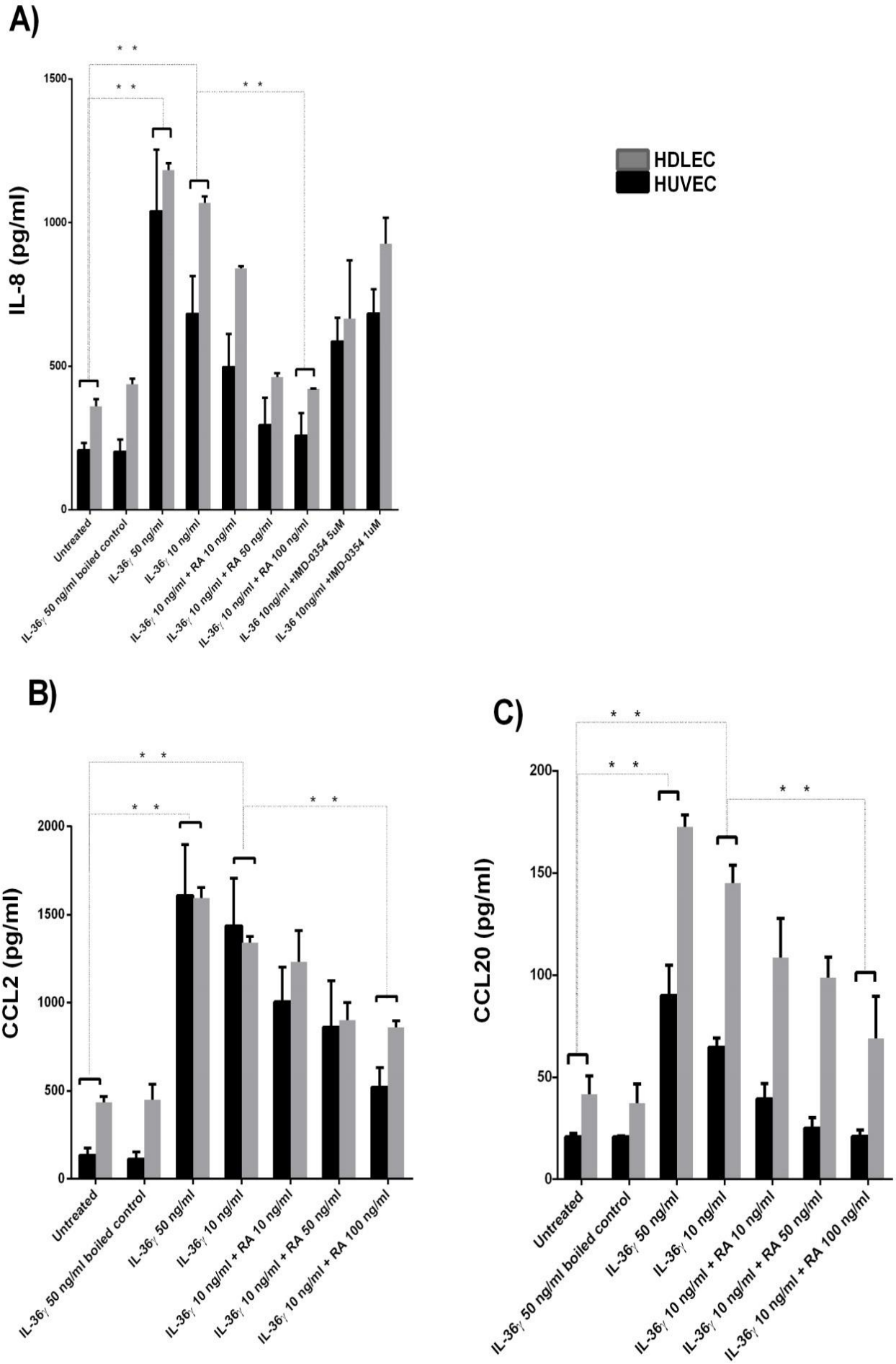

A)
IL-8 (pg/ml)
1500
1000
500
0
* *
* *
* *
HDLEC
HUVEC
Untreated
IL-36γ 50 ng/ml boiled control
IL-36γ 50 ng/ml
IL-36γ 10 ng/ml
IL-36γ 10 ng/ml + RA 10 ng/ml
IL-36γ 10 ng/ml + RA 50 ng/ml
IL-36γ 10 ng/ml + RA 100 ng/ml
IL-36 10ng/ml +IMD-0354 5uM
IL-36 10ng/ml +IMD-0354 1uM
B)
CCL2 (pg/ml)
2000
1500
1000
500
0
* *
* *
* *
Untreated
IL-36γ 50 ng/ml boiled control
IL-36γ 50 ng/ml
IL-36γ 10 ng/ml
IL-36γ 10 ng/ml + RA 10 ng/ml
IL-36γ 10 ng/ml + RA 50 ng/ml
IL-36γ 10 ng/ml + RA 100 ng/ml
C)
CCL20 (pg/ml)
200
150
100
50
0
* *
* *
* *
Untreated
IL-36γ 50 ng/ml boiled control
IL-36γ 50 ng/ml
IL-36γ 10 ng/ml
IL-36γ 10 ng/ml + RA 10 ng/ml
IL-36γ 10 ng/ml + RA 50 ng/ml
IL-36γ 10 ng/ml + RA 100 ng/ml

Figure 19: ECs secrete chemokines following IL-36γ stimulation

Following 48 hr stimulation with IL-36/IL-36Ra or NF-κB inhibitor(IMD), the EC cell supernatant was tested for CCL20, CCL2 and IL-8 by ELISA (A-C). Sample sizes: HUVEC n=5, HDLEC n=3 (3 biological replicates of pooled donors). Standard error of mean depicted. ANOVA *: p<0.05 from relevant controls.

2.4.5 Adhesion molecule upregulation on EC following IL-36γ stimulation

Proinflammatory cytokines are known to upregulate adhesion molecules on ECs thereby facilitating leukocyte recruitment. VCAM-1 is usually undetectable on basal cells, whereas ICAM-1 is detectable at low levels at basal. Both adhesion molecules are upregulated on ECs following proinflammatory cytokine stimulation (Swerlick et al., 1992, Kim et al., 1993).

Our results confirm that IL-36γ also has this ability. FACS analysis confirmed both ICAM-1 and VCAM-1 were upregulated after stimulating ECs with IL-36γ for 48 hr. HUVEC and HDLEC both showed adhesion molecule upregulation in a dose dependent manner. Although stimulating with 10 ng/ml, produced minimal upregulation of adhesion molecules on EC, 50 ng/ml IL-36γ resulted in a clear upregulation of surface expression of both ICAM-1 and VCAM-1. Despite differences in chemokine secretion, no notable difference of adhesion molecule expression was seen between HDLEC and HUVEC.

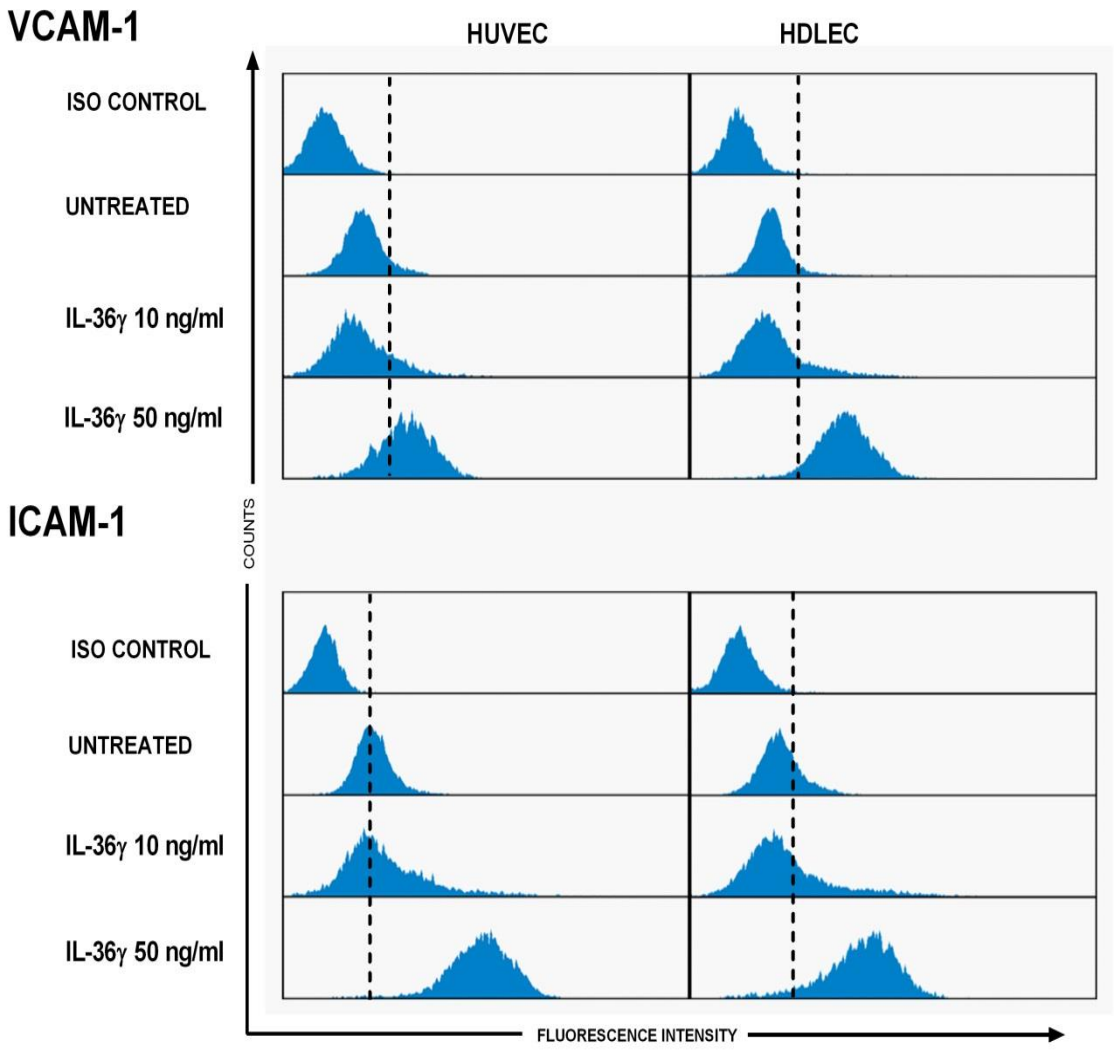


Figure 20: Endothelial cell adhesion molecule upregulation following IL-36γ stimulation

Following 48 hr stimulation with IL-36γ, ECs (HUVEC and HDLEC) were stained with labelled antibodies specific for ICAM-1 or VCAM-1 and analysed by flow cytometry for surface expression. Relevant isotype controls were used as negative control. Fluorescence beyond the "isotype" line in the depicted histograms thus represents specific binding of antibody demonstrating detectable expression. A representative experiment is shown (sample sizes: HUVEC n=3, HDLEC n=3).

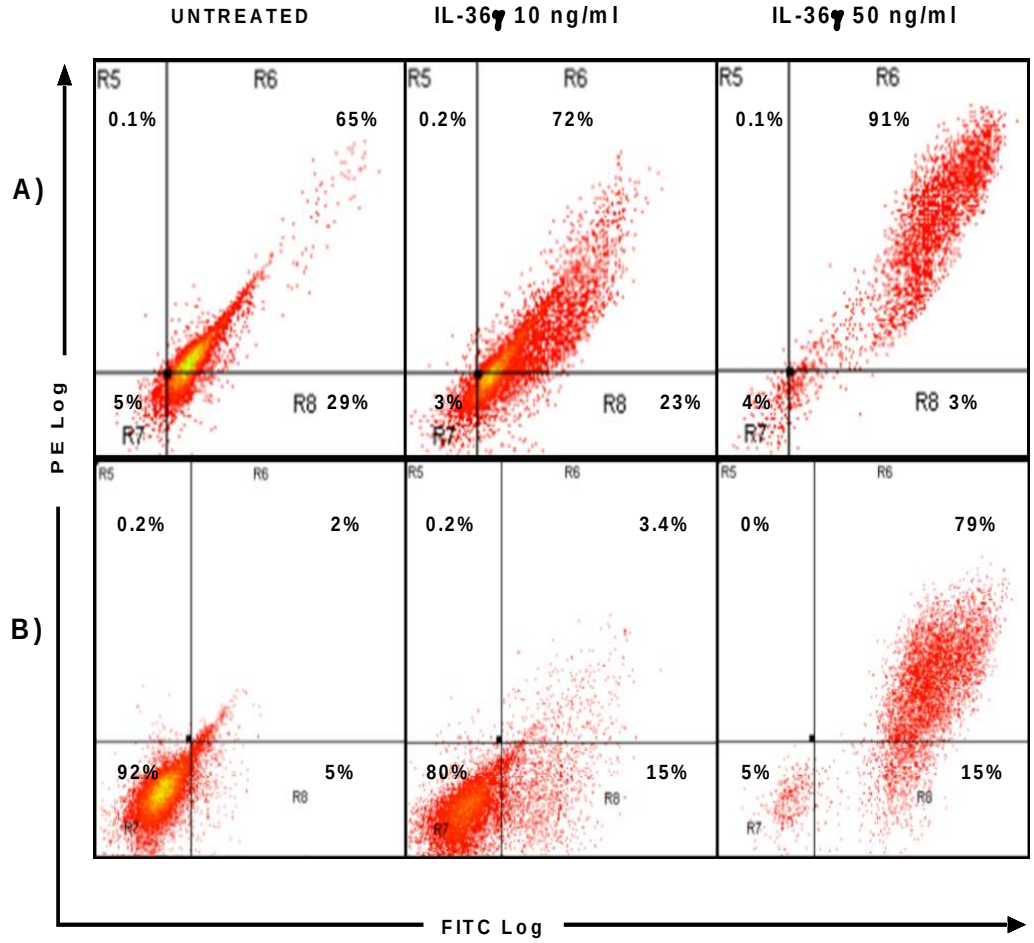


Figure 21: PE log (CD106, VCAM-1) vs FITC log (CD54, ICAM-1), showing surface expression of respective markers following IL-36γ stimulation

For both HUVEC (A) and HDLEC (B), increasing IL-36γ concentrations results in increased double positive cells for both markers (R6 area) when compared to untreated control. n=3, image represent 1 randomly selected experiment.

2.4.6 Supernatant from IL-36γ stimulated ECs is a chemoattractant for T Cells

Having observed chemokine secretion by ECs following IL-36γ stimulation, the chemotactic potency of the EC conditioned media was assessed. CCL20 which was found to be secreted by ECs following IL-36γ stimulation is known to be a chemoattractant for T cells and has a documented role in psoriasis (Homey et al.,

2000). Untreated cell supernatant, media alone and IL-36γ + media were used as negative controls and rhCCL20 as a positive control. Supernatant from IL-36γ stimulated ECs proved to be a chemoattractant for T cells when compared to negative controls (Figure 22). Increasing IL-36γ concentration to 50 ng/ml resulted in a tendency for increased T cell migration. Chemotaxis assays could confirm that IL-36Ra at 100 ng/ml was sufficient to inhibit the stimulatory effects of IL-36γ 10 ng/ml. When stimulating with the Ra at 100 ng/ml, the migration of T cells was reduced and comparable to untreated supernatant. This suggests IL-36γ induced chemokine secretion was indeed responsible for T cell migration.

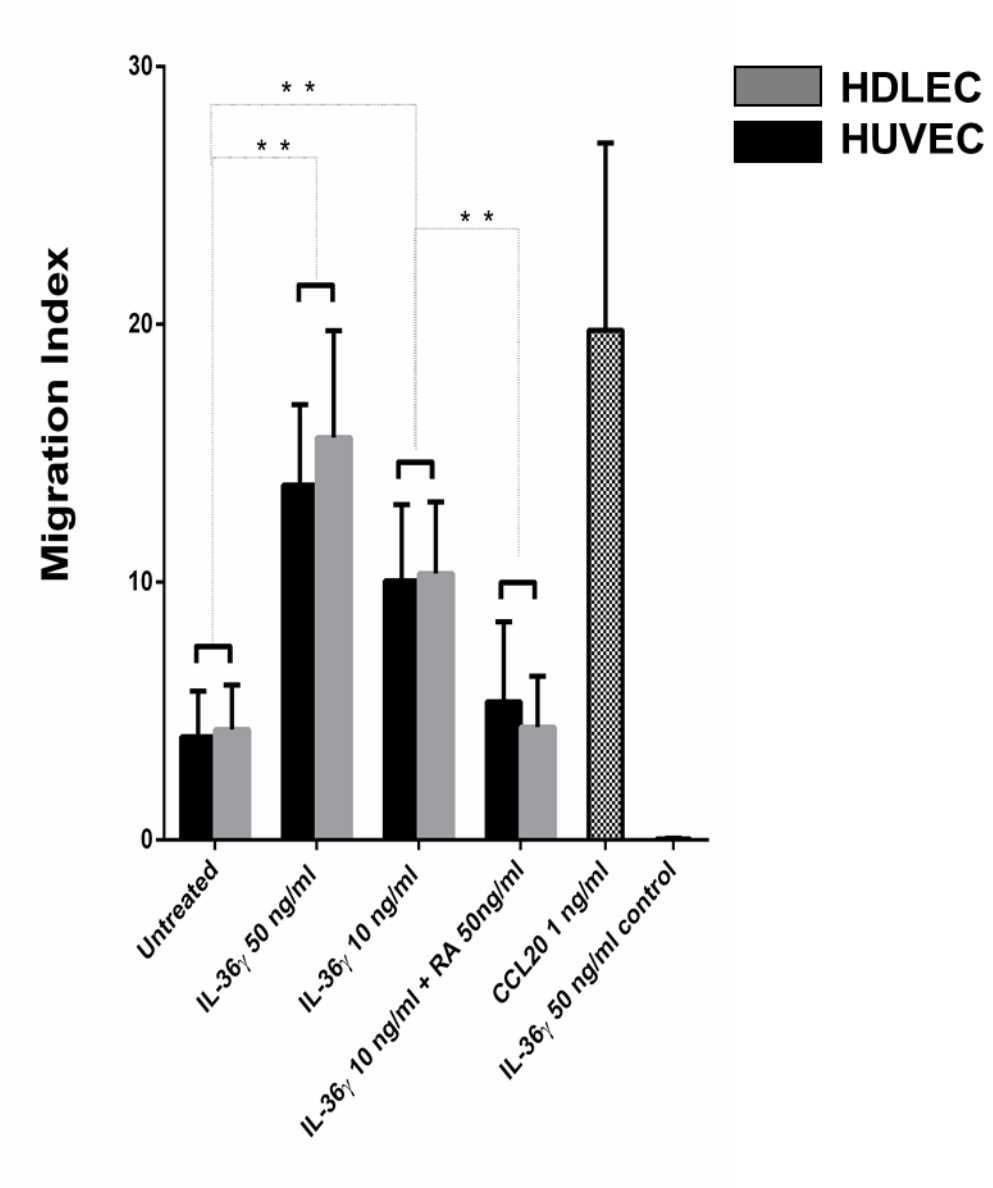


Figure 22: Chemokines secreted by ECs following IL-36 stimulation are a chemoattractant for T cells

The supernatant from treated and untreated ECs was removed and tested for its chemoattractant ability using a chemotaxis assay. The supernatant was placed in the bottom chamber and T cells above. The number of migrated cells in the bottom chamber after 2 hr was then measured. Sample size: n=7. Standard error of mean depicted. ANOVA *: p<0.05 from relevant controls.

2.4.7 IL-36γ stimulation activates NF-kB and c-JUN in ECs

IL-36γ stimulation resulted in increased NF-κB and c-JUN phosphorylation in HUVEC (Figure 23). Concentrations of 10 and 50 ng/ml of IL-36γ resulted in increased detection of the phosphorylated active versions of NF-κB and c-JUN in the nucleus of HUVEC and also in whole protein lysate. The activation of both NF-κB and c-JUN was reduced by the addition of the RA. NF-κB inhibitor only partially inhibited IL-8 secretion suggesting other transcription factors such as AP-1 may also play a role (Figure 19).

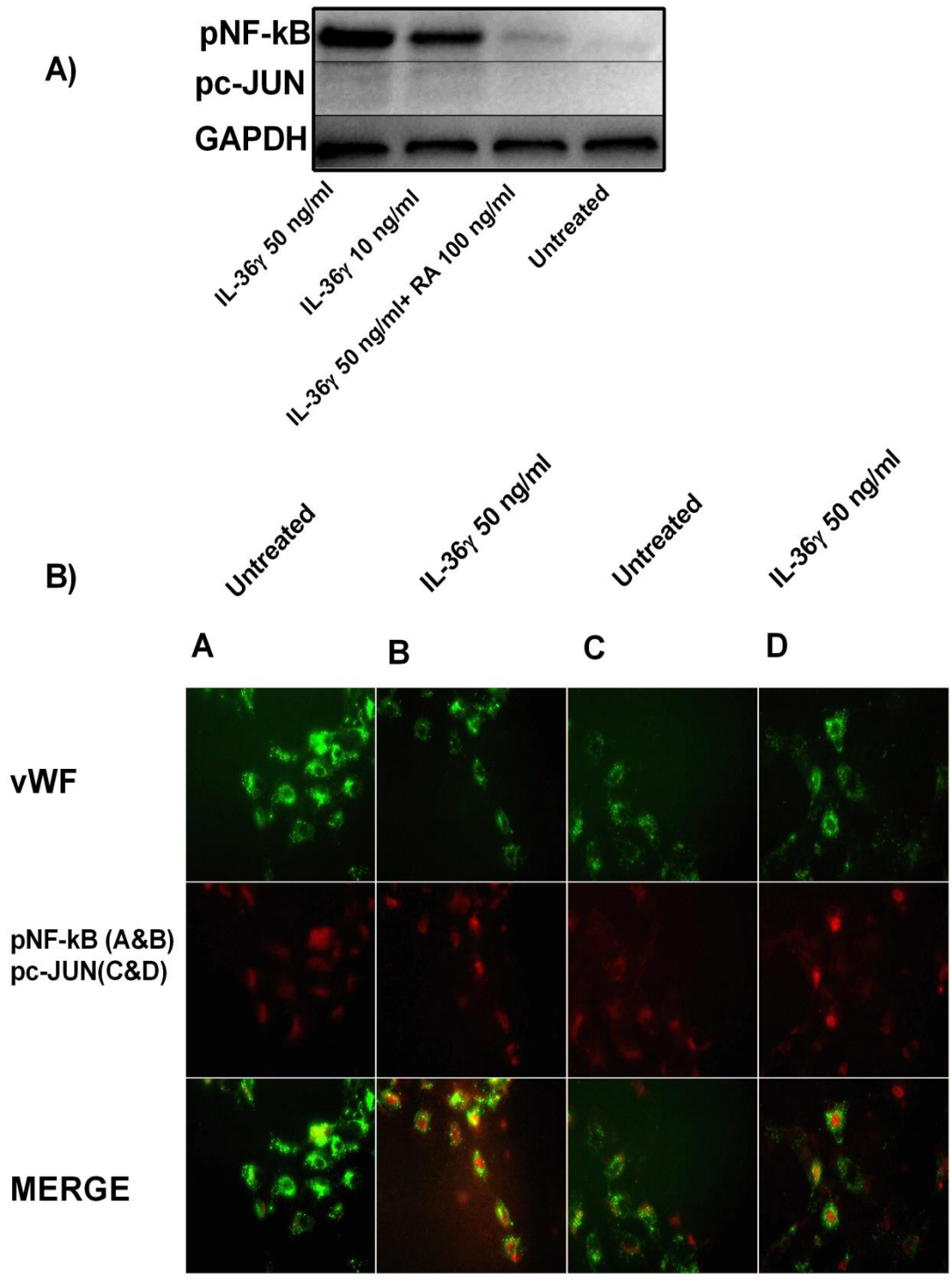


Figure 23: IL-36γ induces NF-κB and c-JUN phosphorylation in HUVEC

Western blot (A) and ICC analysis (B) of pNF-κB-p65 and pc-JUN. Phosphospecific antibodies detect active form of NF-κB or c-JUN in the nucleus of HUVEC. Increased detection of active form of both were also detected in whole cell lysate via Western blot. Magnification x 40.

2.4.8 IL-36γ induces dermal endothelial tube formation

Collaborators had previously shown that IL-36 induces tubule formation of endothelial cells in a co-culture model with fibroblasts, and that this tube formation is dependent on IL-36 induced VEGF from fibroblasts (unpublished). However, this work was conducted on HUVEC.

In a co-culture model of HDLEC and dermal fibroblasts, IL-36y stimulation results in increased tube formation of HDLEC. IL-36γ induced tube formation was blocked by the addition of IL-36Ra and by the VEGF inhibitor Sutent®.

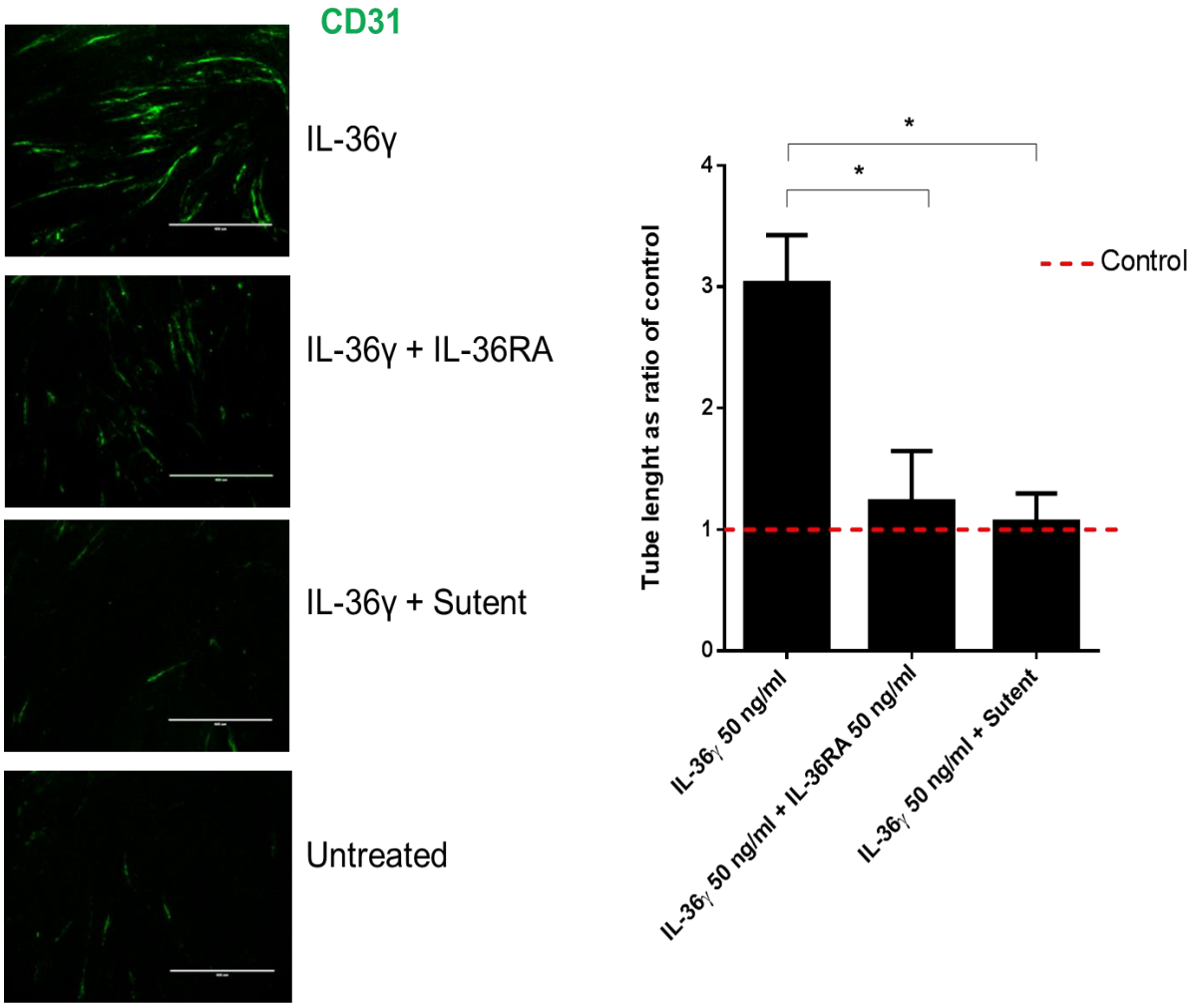


Figure 24: IL-36y induces HDLEC tube formation

HDLEC were co-cultured with DF for 5 days, and stimulated with IL-36, IL-36Ra continuously or Sutent and subsequently stained for CD31 (green). Average tube length was then calculated. One-way ANOVA *: p<0.05 from relevant controls.

2.5 Discussion

Psoriasis is a common chronic inflammatory skin disorder affecting 2-3% of the world population. The IL-1 family member, IL-36 has been linked to psoriasis pathogenesis and disease severity. In inflammatory diseases, ECs are activated by proinflammatory cytokines including, IL-1, TNFα and IL-17, resulting in enhanced leukocyte recruitment and this is well recognised in psoriasis (Heidenreich et al., 2009). However, so far the effects of IL-36 on ECs have been unexplored.

Here, we describe expression of the IL-36 receptor by both large vessel (HUVEC) and microvessel (HDLEC) endothelium. We assessed the functional activity of the receptor by assessing the biologic response of ECs to IL-36γ stimulation, including adhesion molecule upregulation and chemokine production.

qPCR and ICC both confirmed the presence of the receptor on both HDLEC and HUVEC. Interestingly EC expression appeared slightly lower than on both keratinocytes and fibroblasts. Previous research has shown fibroblasts are responsive to IL-36γ and IL-36α resulting in IL-8 secretion (Frey et al., 2013).

The receptor is also known to be highly expressed on keratinocytes when compared to other cell types such as monocytes (Foster et al., 2014). Prior to this study, the IL-36R had also been suggested on a range of EC types through DNA arrays by collaborators (Bridgewood et al., 2017).

HUVEC were initially stimulated with IL-36γ using a range of concentrations and time points. Initial IL-8 secretion data (Figure 18) revealed that the optimal concentrations for stimulating HUVEC were 10 and 50 ng/ml. Stimulating with

100 ng/ml appeared to cause IL-8 secretion to plateau in comparison to 50 ng/ml. Stimulating with 5 ng/ml resulted in no statistically significant increase in IL-8 secretion when compared to untreated controls. Stimulating with 10 ng/ml only resulted in a statistical significance when at 48 hr. For all concentrations, IL-8 secretion increased compared to untreated control when stimulated at 48 hr. Previous research with IL-36γ stimulations and dermal fibroblasts also found IL-8 secretion to be most measurable at 48 hr (unpublished work, Wittmann, Leeds group). For all following read outs of chemokine production and adhesion molecule expression, 48 hr stimulations were used with the concentrations of 10 and 50 ng/ml IL-36γ.

Adhesion molecules expressed on ECs are involved in leukocyte extravasation, by allowing leukocytes to bind them, and allowing migration to the site of inflammation. Both adhesion molecules ICAM-1 and VCAM-1 were upregulated following IL-36γ stimulation. Adhesion molecules are involved in leukocyte extravasation, which allows the movement of leukocytes to the site of tissue damage or infection. Selectins are responsible for the rolling and capture of leukocytes whereas ICAM-1 and VCAM-1 are involved in the adhesion and transmigration. The integrins expressed on leukocytes, LFA-1 and VLA-4 bind to ICAM-1 and VCAM-1 respectively (Muller, 2013). In the context of psoriasis pathogenesis, both these adhesion molecules are required for T cell adhesion and migration and are upregulated in lesions (de Boer et al., 1994, Gabay and Towne, 2015, Blumberg et al., 2010).

ECs also responded with chemokine production to IL-36γ stimulation. CCL2 (MCP1) has documented importance in psoriasis and other inflammatory diseases by recruiting monocytes (Lembo et al., 2014). IL-8, which promotes

neutrophil recruitment, EC survival and angiogenesis, was also secreted following IL-36γ stimulation (Li et al., 2003). The importance of angiogenesis in psoriasis is well recognised (Heidenreich et al., 2009), and local EC secretion of IL-8 induced by IL-36γ could be a contributing factor. CCL20 was secreted which binds to CCR6, which is highly expressed on IL-17 and IL-22 producing lymphocytes and the majority of T cells that infiltrate the skin in psoriasis are CCR6 positive (Homey et al., 2000). Serum levels of CCL20 have been suggested to correlate with psoriasis severity (Ekman et al., 2013). Although keratinocytes are a main source of CCL20 (Homey et al., 2000, Teraki et al., 2004) our findings indicate that ECs could contribute to the cutaneous CCL20 as well as CCL2 production.

For all chemokines analysed, the IL-36Ra was able to dampen the effect in a dose dependent manner. This finding was also mirrored in the chemotaxis assay where T cell migration was reduced when ECs had been co-stimulated with the Ra.

While the role of TNFα is well characterised in psoriasis, it is hypothesized environmental triggers can cause keratinocytes to secrete IL-36γ independently of proinflammatory cytokines such as TNFα (Gabay and Towne, 2015, Blumberg et al., 2010). It is thus possible that IL-36γ could be important in the initiation of EC activation, but results in this study suggest its on-going secretion could contribute to chronic EC activation, contributing to the chronic inflammatory nature of the disease.

Our presented findings could also have implications for other diseases. Lung bronchial epithelial cells secrete IL-36 in response to cytokines such as TNFα

(Chustz et al., 2011). Cigarette smoke, the causative agent of Chronic Obstructive Pulmonary Disease (COPD) is also known to cause IL-36 secretion in bronchial epithelial cells (Parsanejad et al., 2008). However, the exact role of IL-36 in respiratory disease it still unknown. In COPD, like many chronic inflammatory diseases, angiogenesis and EC activation occurs (Siafakas et al., 2007). Therefore IL-36γ may also have a role in EC activation and leukocyte recruitment in COPD. Similarly, IL-36β has been linked to disease severity of periodontal inflammation (Kursunlu et al., 2015). Murine models of liver damage/liver inflammation have also identified enhanced expression of IL-36γ, and elevated CCL20 levels, which could be from ECs (Scheiermann et al., 2015).

Collaborators from the University of Leeds have also established that IL-36 promotes angiogenesis by inducing VEGF secretion from surrounding fibroblasts in co-culture models (unpublished). However, this study was conducted using HUVEC, and we report similar findings using the skin relevant HDLECs. VEGF and VEGFR are overexpressed in psoriasis lesions and serum levels of VEGF correlate with PASI (Young et al., 2004, Nofal et al., 2009, Detmar et al., 1994). A SNP in VEGF is also associated with severe psoriasis (+405 CC) and is thought to result in increased VEGF production (Diaz et al., 2000, Young et al., 2006). Interestingly the SNP is also associated with poor prognosis in patients with chronic heart failure (van der Meer et al., 2005). Importantly, it is thought angiogenesis is induced before the onset of the lesion, so it could be hypothesised, that IL-36γ released from damaged keratinocytes would be well placed to induce VEGF and thus angiogenesis when compared to other cytokines that would have importance in a chronic lesion (Telner and Fekete, 1961).

Angiogenesis has even been muted as a potential therapeutic target for psoriasis (Heidenreich et al., 2009, Guérard and Pouliot, 2012, Weidemann et al., 2013). Case reports have demonstrated improvements in psoriatic disease severity scores (PASI) through targeting pro-angiogenic factors such as VEGF-A. Bevacizumab, a monoclonal antibody against VEGF-A used in the treatment for solid cancers, has also been shown to be effective in treating psoriasis, including one case of complete remission for a patient being treated for metastatic colon cancer (Akman et al., 2009). Case reports for tyrosine kinase inhibitors that target VEGFR such as sunitinib (Sutent®) and sorafenib also have produced positive results regarding psoriasis remission (Narayanan et al., 2010, Fournier and Tisman, 2010, Antoniou et al., 2016). Of interest, G6-31, a murine antibody against VEGF-A has demonstrated therapeutic improvement, in a mouse model of psoriasis (Schonthaler et al., 2009). VALPHA is a fusion protein that targets both TNFα and VEGF and has shown to be effective in treating TPA induced psoriasis in mouse models (Jung et al., 2011).

IL-36γ serum levels are enhanced in psoriasis patients (D'Erme et al., 2015). In recent years psoriasis has been suggested as an independent risk factor for atherosclerosis (Gelfand et al., 2006). In atherosclerosis, endothelial activation and initial inflammatory signalling represent an important stage of disease development (Hajra et al., 2000). Increased serum levels of IL-36γ and therefore possible increased endothelium activation within the arteries could accelerate atherosclerotic disease progression. Also of concern, GPP is associated with a Ra mutation, which results in reduced control over IL-36. However the risk factor of GGP on CVD is largely unexplored so far.

These results suggest that IL-36γ has a role in activating the endothelium further enhancing lymphocyte recruitment and thus enhancing psoriatic inflammation. These findings add to the growing importance of IL-36γ in both psoriasis initiation and maintenance. Further studies will help decipher the significance of IL-36γ and its interactions with other EC stimulating mediators such as TNFα and IL-17 in human psoriatic tissue.

3) IL-36 relationship with Psoriasis Macrophages

As defined in the introduction, both IL-36 and macrophages play pivotal roles in the pathogenesis of psoriasis. However, the relationship between IL-36 and macrophages is yet to be fully deciphered.

Hypothesis

I hypothesize that macrophages will be stimulated by IL-36γ and that this will lead to the secretion of disease relevant cytokines important to psoriasis pathology. It is also likely that macrophages from psoriasis patients may have increased secretions of these cytokines due to several genetic immune predispositions known to exist in psoriasis. Finally, I wish to test the hypothesis that this macrophage activation will activate the endothelial cells and lead to increased leukocyte adhesion.

3.1 Aims

Examine the stimulatory effects of IL-36γ + IFNγ on both healthy and psoriasis macrophages by measuring levels of psoriasis important cytokine such as IL-23 and TNFα

- Determine the significance of these inducible mediators on the surrounding tissues by stimulating HUVEC with macrophage supernatant
- Determine if psoriasis monocytes show increased adhesion to a stimulated and resting endothelium
- Determine possible mechanisms behind any adhesion trends such as role of CX3CL1

3.2 Methods

3.2.1 Cell isolations and cell culture

Psoriasis patient blood was obtained under ethical approval (PDAR study: REC 16/YH/0086) and approved by Yorkshire and the Humber Leeds West Research Ethics Committee. Written informed consent was obtained from all subjects. Patients had a PASI score from 0-15. Patients on biologic treatment were used for experiments; however, patients on immunosuppressive treatment such as methotrexate were not.

Blood was collected in sodium citrate tubes. PBMCs were separated using Lymphoprep density gradient centrifugation. The mononuclear cells were collected and centrifuged at 200xg for 10 mins to remove platelets. The cells were counted and resuspended in 80 µl of 1% BSA in PBS per 1 $x10^7$ cells. 20 µl of CD14+ microbeads (Miltenyi Biotech) were added per 1 $x10^7$ cells. Cells were incubated for 15 mins and centrifuged at 300xg and resuspended in 500 µl 1% BSA in PBS. Monocytes were isolated from PBMCs using the Dynal MPC column (Invitrogen, California, USA). Monocytes were resuspended in RPMI (Thermofisher Scientific, Massachusetts, USA) containing 10% FCS containing penicillin/streptomycin (100 U/100 mg/mL), (both Life Technologies, Carlsbad, USA).

Umbilical cords were supplied by Bradford Royal Infirmary under the approval and processing of Ethical Tissue Bradford. HUVECs (human umbilical vein endothelial cells) isolated from umbilical cords in a previously described method. Cells were incubated in an atmosphere of 95% humidity and 5% CO2 at 37°C.

3.2.2 Macrophage purity and IL-36R confirmation

Macrophages isolated as previously described, by seeding isolated monocytes onto coverslips overnight. Cells were washed in PBST and fixed in 4% formaldehyde for 20 mins. Cells were then blocked for 1 hr in 5% BSA in PBS. Cells were incubated overnight with rabbit anti-human IL-36R 1:500 (Novus Biologics, Littleton, USA) or Rabbit IgG isotype control (1:500) (Abcam, Cambridge, UK). Cells were then washed with PBST and incubated with donkey anti-rabbit Alexa 594 conjugated and mouse anti-human CD14 FITC conjugated or mouse IgG isotype control (both 1:100) (both ImmunoTools, Gladiolenweg, Germany). Cells were visualised using the EVOS XL microscope (Hatfield, PA, USA).

3.2.3 Macrophage cytokine stimulation

Monocytes were seeded at $1x10^5$ in 96 well plates (Greiner Bio-One, Stonehouse,UK) in RPMI overnight to generate day 1 macrophages. Macrophages were stimulated with either IL-36γ protein which was generated as previously described (Ainscough et al., 2017), IL-17, TNFα, IL-1α w/wo IFNγ (PeproTech, Rocky Hill, NJ, USA). Following 48 hr stimulation, supernatant was stored at -80 °C. Concentrations of IL-23 and TNFα were measured using ELISA kits from eBioscience/Thermofisher (Waltham, MA, USA). ELISAs were carried out according to the manufacturer's protocols. Reproducibility of the supernatants results was confirmed by triplicate testing, with <10% error.

3.2.4 Macrophage supernatant stimulation of EC ICAM-1 expression

Following 48 hr stimulation of macrophages, supernatant was removed and centrifuged and stored at -80°C. HUVEC were cultured on black TC grade

fluorescence plates (PerkinElmer, Waltham, MA, USA), in complete Promocell endothelial cell media containing penicillin/streptomycin (100 U/100 mg/mL). Supernatant diluted 1:10 with media and used to stimulate HUVEC for 24 hr. Recombinant IL-36 was added to HUVEC to serve as control wells/blank. After 24 hr, the cells were fixed for 15 min with 4% formaldehyde in PBS. Mouse anti-human ICAM-1 FITC or mouse IgG isotype control was added (1:500) (BioLegend, San Diego, USA). The fluorescence intensity of each well was measured using the Promega GloMax plate reader (Madison, WI, USA). For immunocytochemistry, the cells were visualised using the EVOS XL microscope.

3.2.5 Monocyte/HUVEC adherence assays

Monolayers of HUVEC were grown to confluence in 24 well plates (Greiner Bio-One). EC were stimulated with macrophage supernatant as above, or with TNFα (10 ng/ml)(positive control) for 24 hr and then suspended in fresh media prior to experiments. Where relevant, a blocking antibody against CX3CL1 (R&D system, Abingdon, UK) was added at 100 ng/ml for 1 hr prior to experiment before the replacement of media. 1x 10^5 monocytes were added per chamber for 30 mins. After 30 mins, non-adherent cells were washed away and the cells were fixed using 4% formaldehyde in PBS and blocked in 5% BSA in PBS for 1 hr. The cells were the stained with mouse anti-human CD14 FITC conjugated or mouse IgG isotype control (both 1:100) (both ImmunoTools). The cells were visualised using the EVOS XL microscope and the number of adhered monocytes counted using ImageJ software plugin.

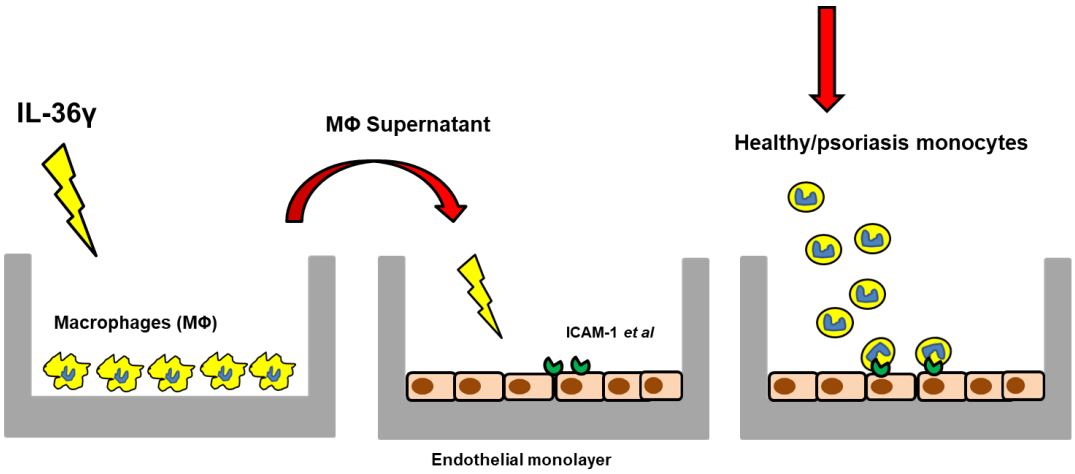


Figure 25: Depiction of methods

IL-36 stimulated macrophage supernatant (48 hr) is harvested and subsequnetly used to stimulated EC monolayer (24hr). Following this, ICAM-1 expression is measured or monocytes are allowed to adhere.

3.2.6 Statistical analysis

Statistical significance was calculated using a one-way ANOVA with a Bonferroni's multiple comparisons test, unless otherwise stated. Analysis was performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Error bars represent the standard error of the mean (SEM). *: $p<0.05$ from indicated controls.

3.3 Results

3.3.1 Macrophages express IL-36R

Whilst IL-36R had been confirmed at mRNA level on macrophages and by psoriasis tissue histology, it had never been previously confirmed on isolated macrophages and at the protein level (Boutet et al., 2016). Contaminating

lymphocytes may influence downstream readouts, such as cytokine secretion, so therefore CD14+ staining confirms the purity of the cell population.

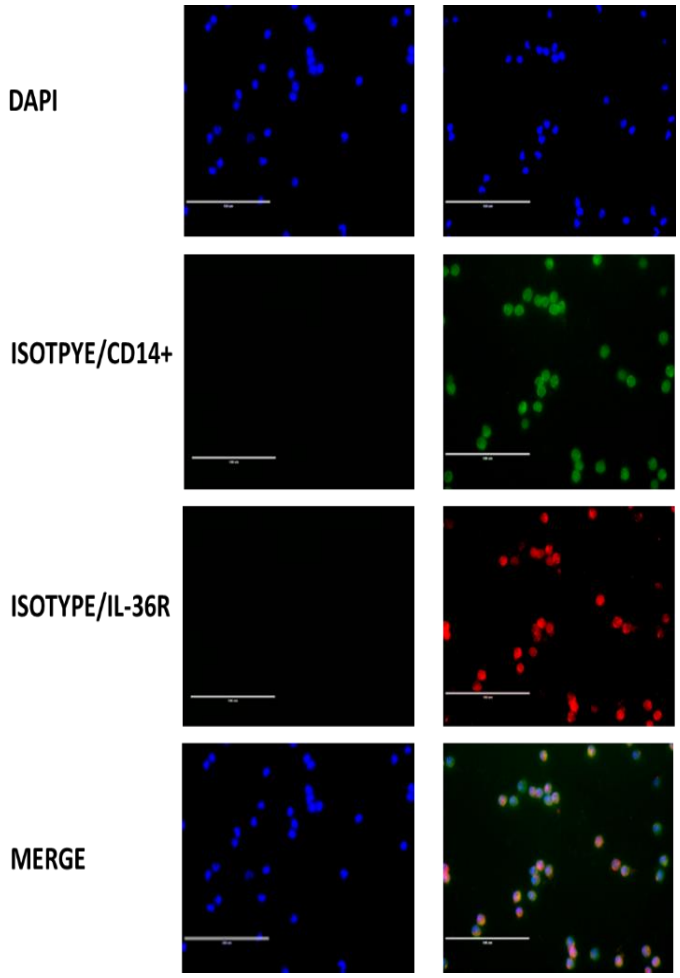


Figure 26: Macrophages express IL-36R

Isolated macrophages were stained for either IL-36R (RED) or also CD14+ (Green) to confirm cell purity. Isotype controls confirm specific binding of antibodies. Magnificent X 20.

3.3.2 IL-36 has proinflammatory effects on macrophages

Whilst previous research has suggested monocytes respond to IL-36 by secreting inflammatory mediators, little is known about IL-36γ kinetics of macrophage stimulation. Healthy patient macrophages were stimulated with a range of concentrations of IL-36γ and time points, to identify the most effective stimulation

for TNFα secretion. Statistical significance compared to untreated was achieved by IL-36γ concentrations of 10 and 50 ng/ml for 48 hr but not 24 hr as seen in figure 27. No statistical significance was achieved for IL-36γ 1 ng/ml at either 24 or 48 hr time point. Concentrations of over 100 ng/ml may lead to increased secretions, but raises questions over physiological relevance.

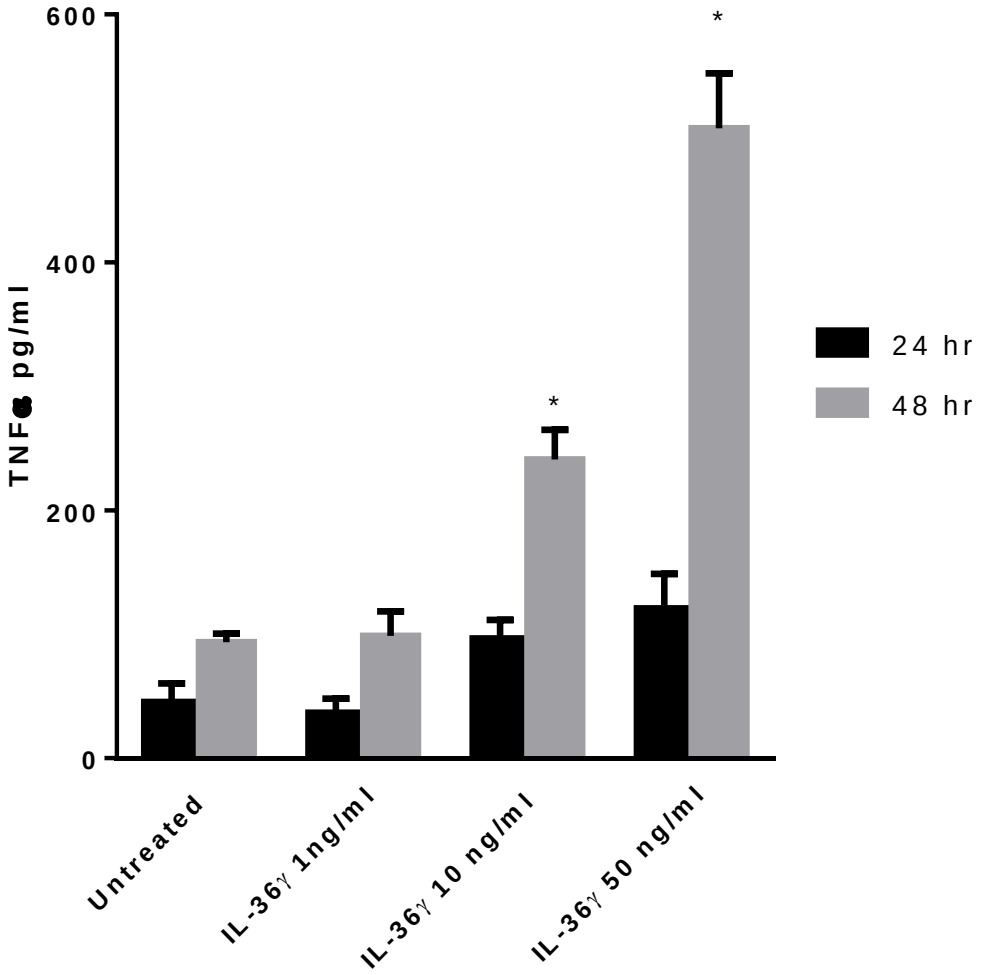


Figure 27: IL-36γ induced TNFα secretion from healthy macrophages

TNFα secretion from healthy macrophages stimulated with IL-36γ 1-100 ng/ml at both 24 and 48 hr. Sample size, n=4. ANOVA *: p<0.05 from relevant controls.

3.3.3 IFNγ activates macrophages and enhances IL-36γ induced TNFα secretion.

IFN-γ has a documented importance in macrophage activation (Hu and Ivashkiv, 2009), however its ability to enhance IL-36γ induced secretions is presently unknown. Macrophages were primed with increasing concentrations of IFNγ for 24 hr before being stimulated with IL-36γ at 10 ng/ml. TNFα secretion into the media was measured at both 24 and 48 hr. Statistical significance was achieved when IL-36γ (10 ng/ml) at 48 hr was primed with IFNγ 20 ng/ml which appeared to plateau when increasing to IFNγ 100 ng/ml. Following preliminary stimulation data, it was thus decided that macrophages would be stimulated with both IL-36γ 10 and 50 ng/ml and with and without IFNγ 20 ng/ml.

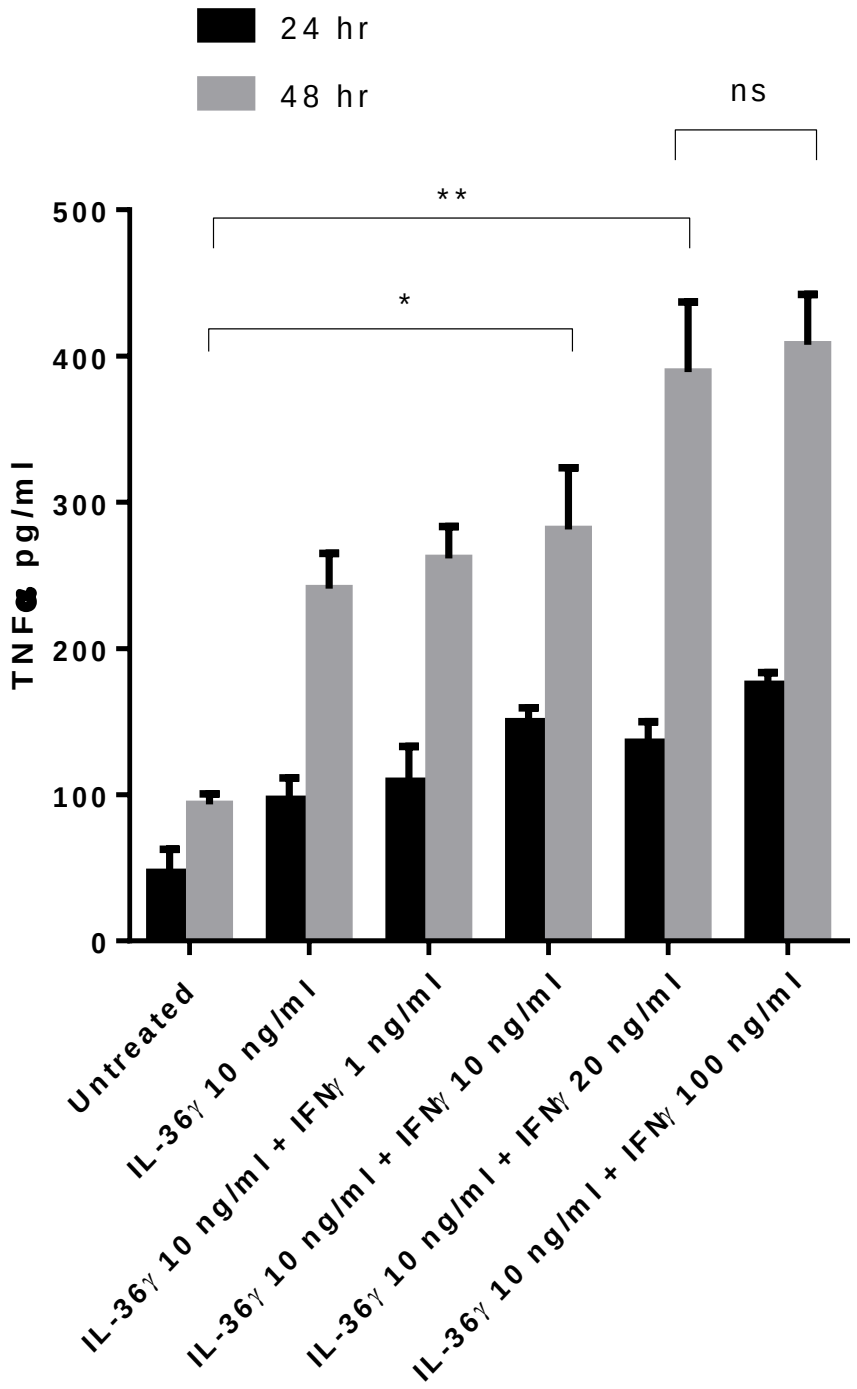
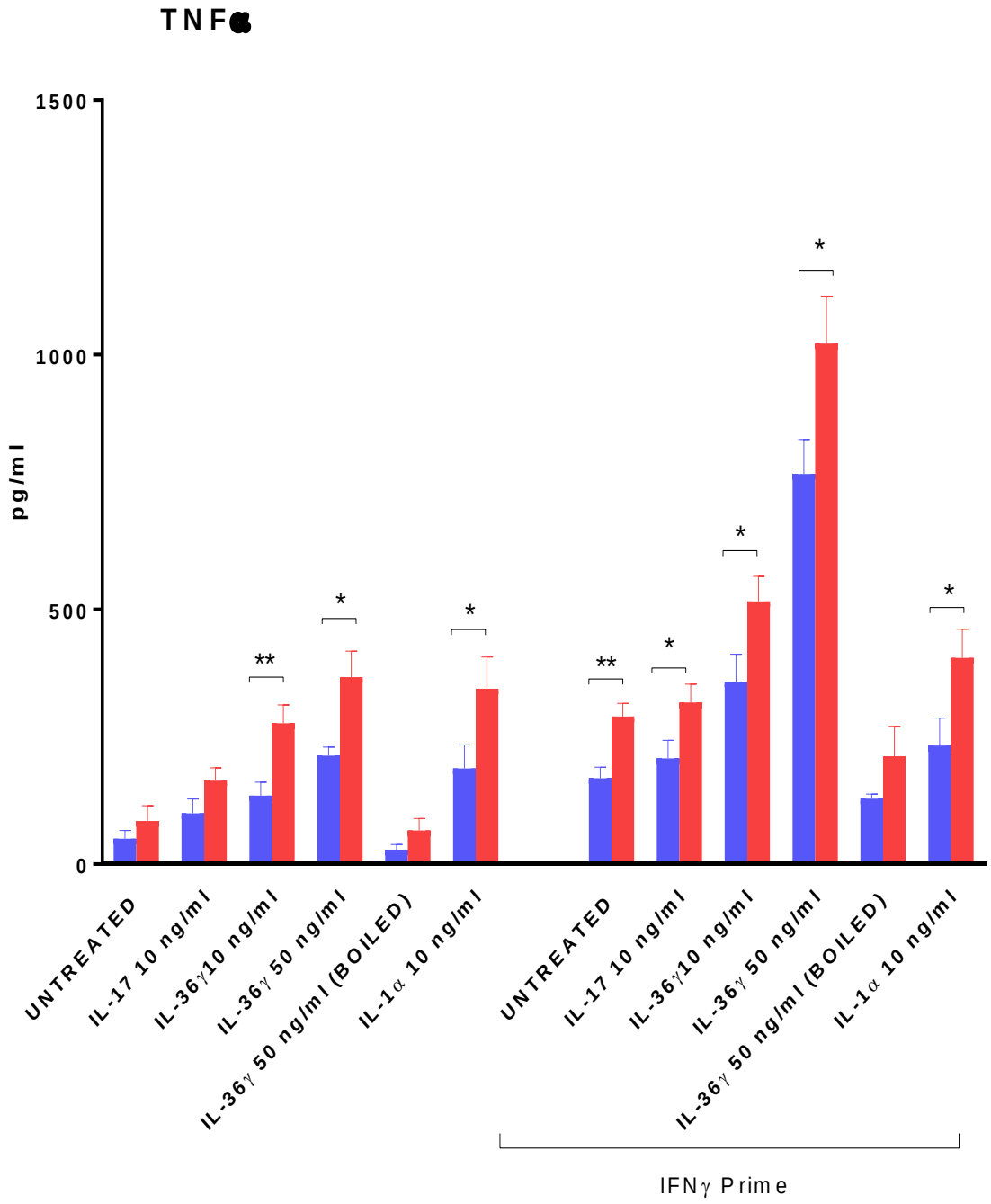


Figure 28: IL-36γ induced TNFα secretion from healthy macrophages is enhanced by IFNγ

TNFα secretion from healthy macrophages stimulated with IL-36γ 10 ng/ml and primed with increasing concentrations of IFNγ. Sample size, n=3. ANOVA *: p<0.05 from relevant controls.

3.3.4 IL-36 induces increased IL-23 and TNFα from psoriasis macrophages when compared to healthy macrophages

To assess the functional significance of IL-36γ stimulation of macrophages in the context of a psoriasis lesion, macrophages were stimulated with IL-36γ for 48 hr and the levels of TNFα and IL-23 measured by ELISA. Macrophages are known to be sensitive to LPS stimulation so to confirm no contamination was present, boiled IL-36γ was included. Both 10 and 50 ng/ml IL-36γ were able to induce a significant increase in IL-23 secretion when compared to unstimulated (Figure 29), which was further amplified when the macrophages were primed with IFNγ 20 ng/ml. For both IL-36γ 10 and 50 ng/ml, psoriasis macrophages secreted significantly more IL-23. Treatment with macrophages with other cytokines with a known presence within psoriasis lesions such as IL-17, TNFα and IL-1 were unable to induce a significant increase in IL-23 secretion, regardless of IFNγ priming. IL-36y also induced significant TNFα secretion from macrophages, as did both IL-1 and IL-17 when compared to untreated controls. However following IFNγ priming, IL-36γ induced secretion exceeded both IL-1 and IL-17.


TNFα
1500
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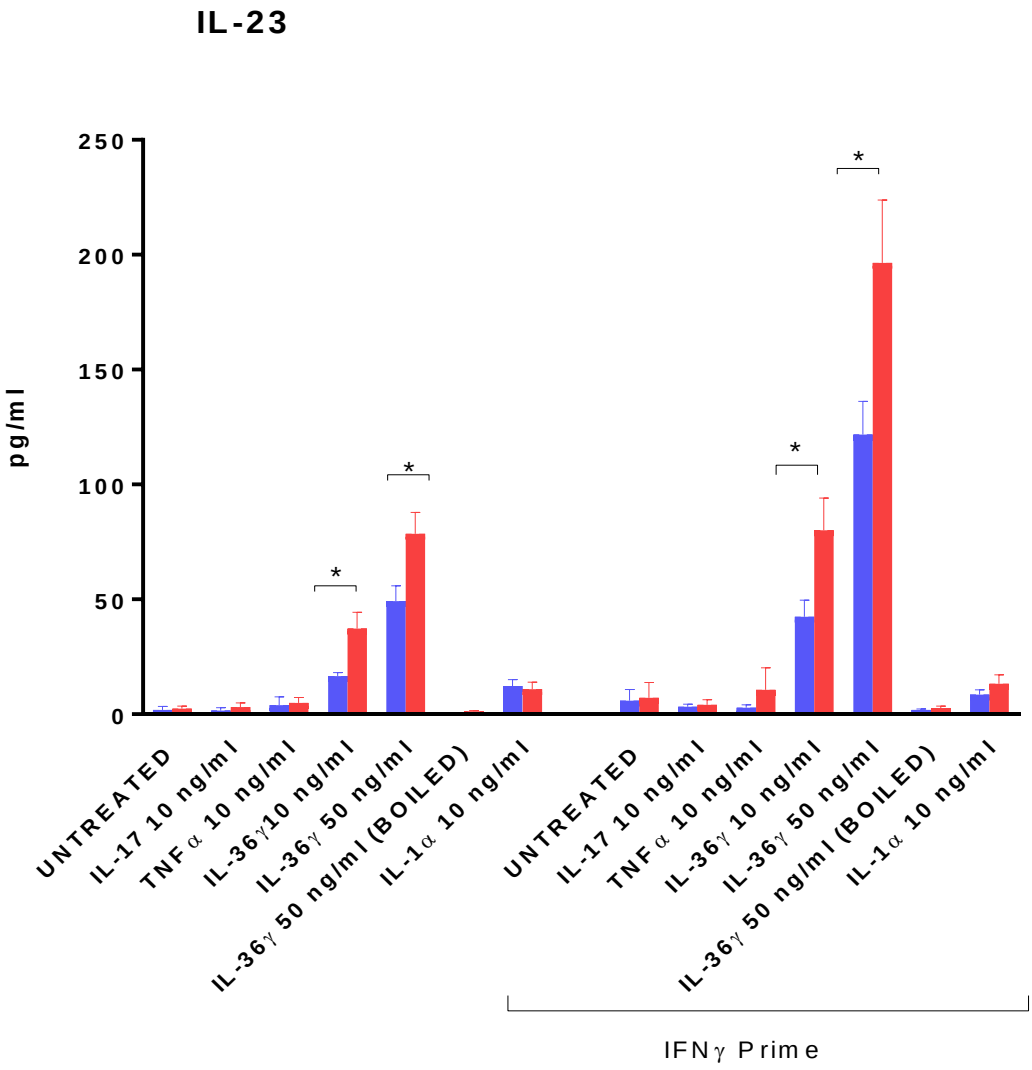

Figure 29: IL-36γ induced TNFα and IL-23 secretion from psoriasis and healthy macrophages

Macrophages were primed w/wo IFNγ and stimulated with various cytokines (IL-17, TNFα, IL-36γ and IL-1) and subsequent IL-23 and TNFα secretion was measured following 48 hr. **Unpaired t test*: *$p<0.05$ from relevant controls.***

3.3.5 IL-36γ induced macrophage supernatant is inflammatory to endothelial cells

To assess the functional significance of IL-36 induced macrophage cytokines on surrounding tissues, we stimulated HUVEC with macrophage supernatant. To identify the most sensitive concentration to stimulate HUVEC with, both untreated and IL-36 50 ng/ml induced supernatant (treated) was diluted in media from concentrations ranging from neat to 1:1000. To assess the proinflammatory effects of macrophage supernatant on HUVEC, ICAM-1 upregulation was measured by fluorescence intensity. For both 1:1000 and 1:100 dilutions, no significant change in ICAM-1 expression was seen for untreated versus treated. For 1:10, treated ICAM-1 expression was significantly higher than untreated. For neat, no statistical significance was seen between treated and untreated.

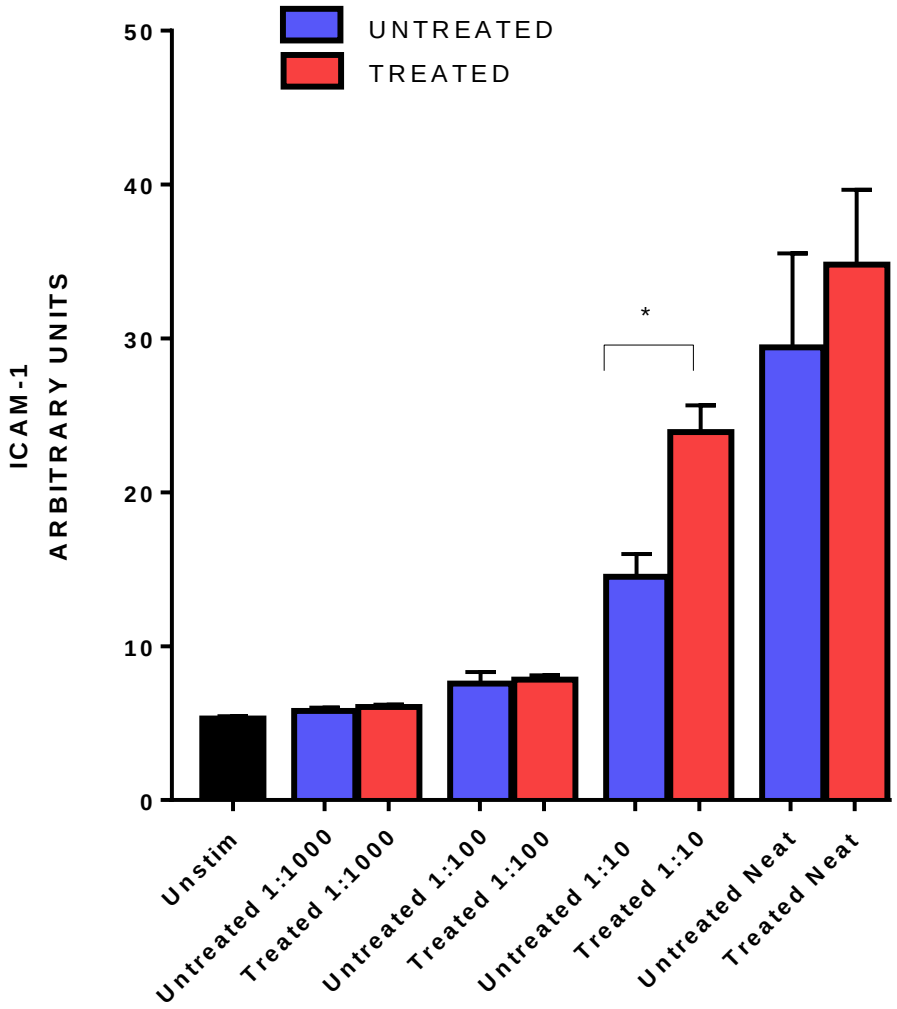

Figure 30: Macrophage supernatant is proinflammatory to HUVEC

HUVEC were stimulated with various concentrations of healthy macrophage supernatant and ICAM-1 intensity of measured. n=3. Unpaired t test *: p<0.05 from relevant controls.

3.3.6 Psoriasis macrophage derived supernatant shows greater propensity to upregulate ICAM-1

After this optimisation where healthy macrophage supernatant was used to determine activation of HUVEC as measured by ICAM-1 expression, HUVEC were stimulated with both psoriasis and healthy macrophage derived supernatant (both IL-36 treated and untreated macrophages). For both IL-36 10 and 50 ng/ml

induced supernatant, psoriasis derived macrophage supernatant showed increased activation of ICAM-1 on HUVEC (Figure 31). Isotype control was included to ensure ICAM-1 binding was specific. ICAM-1 staining also confirms increased upregulation when stimulated with with psoriasis macrophage derived supernatant (Figure 32). IL-36 may itself upregulate ICAM-1, recombinant IL-36 10 and 50 ng/ml were used to zero expression (not shown).

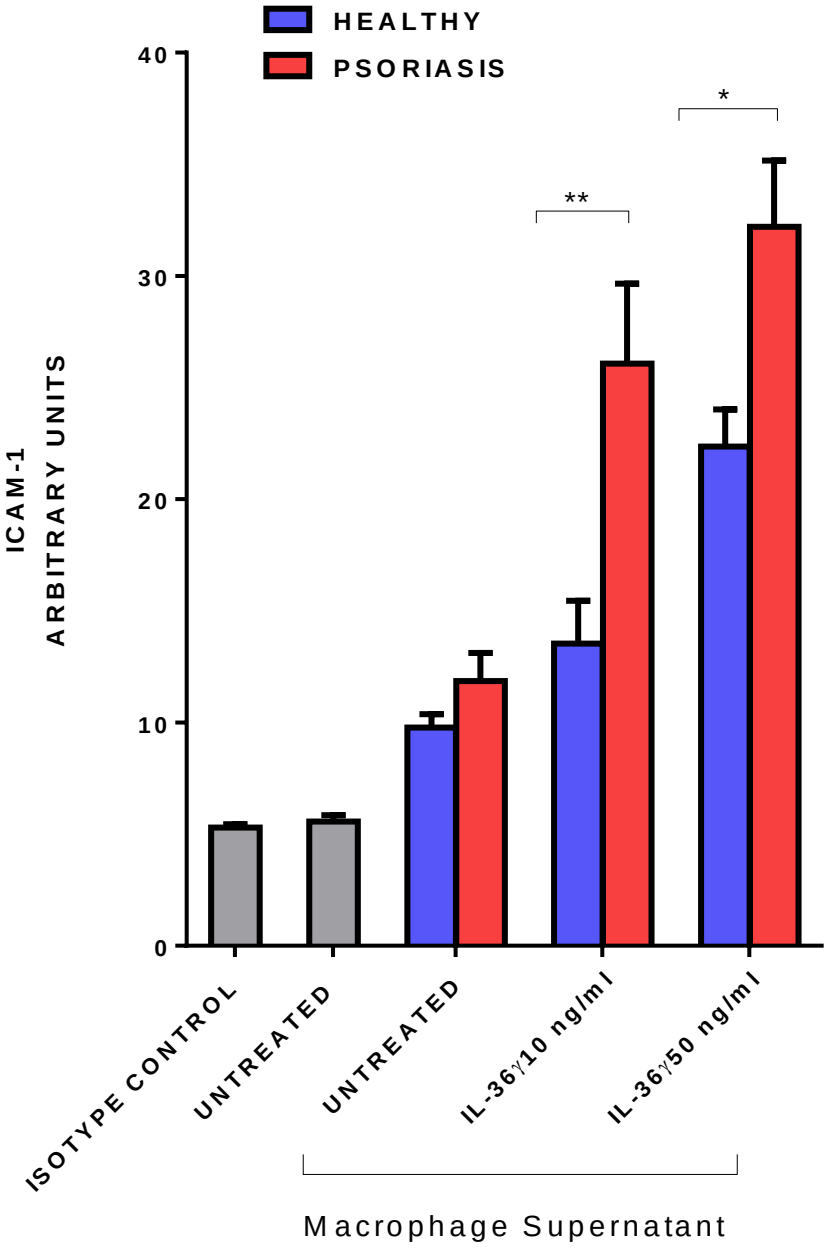

Figure 31: Psoriasis derived macrophage supernatant significantly upregulates ICAM-1 compared to healthy

HUVEC were stimulated with supernatant from both healthy and psoriasis macrophages treated with IL-36 for 24 hr and ICAM-1 intensity measured. (Psoriasis n=8, Healthy n=8). Unpaired t test *: p<0.05 from relevant controls.

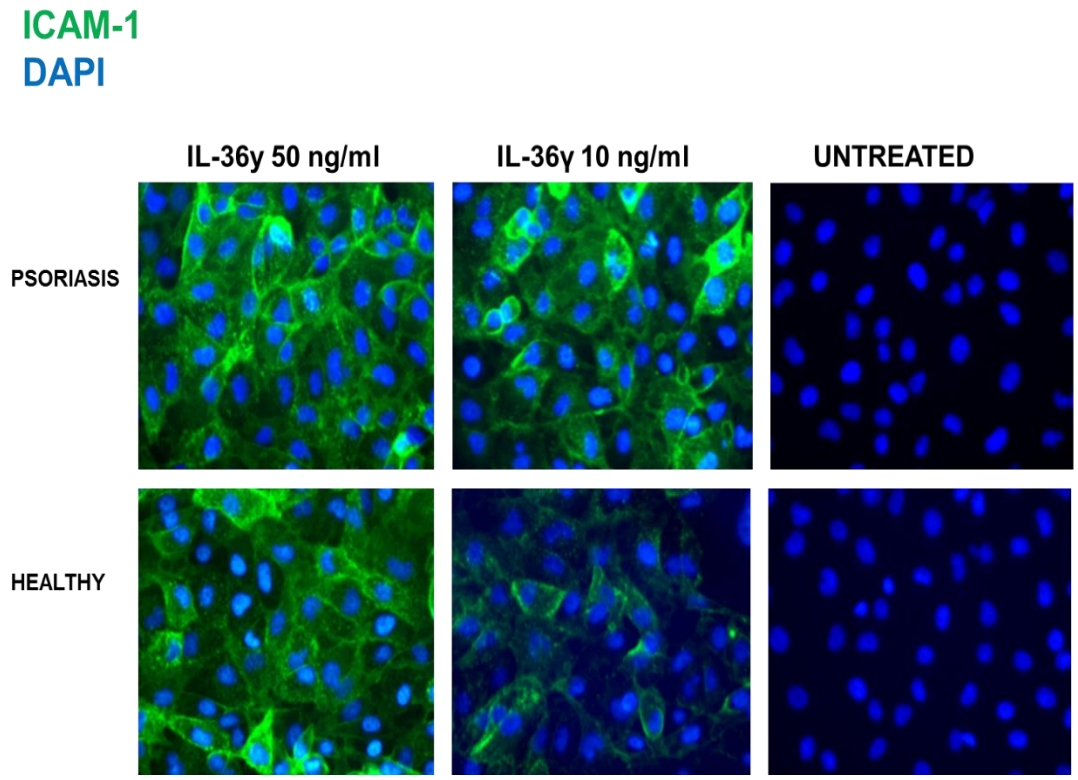

Figure 32: Psoriasis derived macrophage supernatant significantly upregulates ICAM-1 compared to healthy

HUVEC stimulated with either psoriasis or healthy derived macrophage supernatant for 24 hr and ICAM-1 expression visualised by ICC. Magnification x 40.

After finding that psoriasis-derived macrophage supernatant had greater inflammatory potential than healthy macrophage supernatant, we next assessed the functional significance of this in the context of immune cell recruitment. Monocytes from both healthy and psoriasis patients were isolated and allowed to adhere to stimulated HUVEC. HUVEC were either stimulated with macrophage supernatant (untreated and IL-36-induced) or TNFα 10 ng/ml (positive control), for 24 hr. For all treatments, psoriasis monocytes showed increased adherence compared to healthy monocytes. In agreement with psoriasis macrophage

supernatant showing increased endothelial activation ability, monocytes showed increased adherence to this treated endothelium.

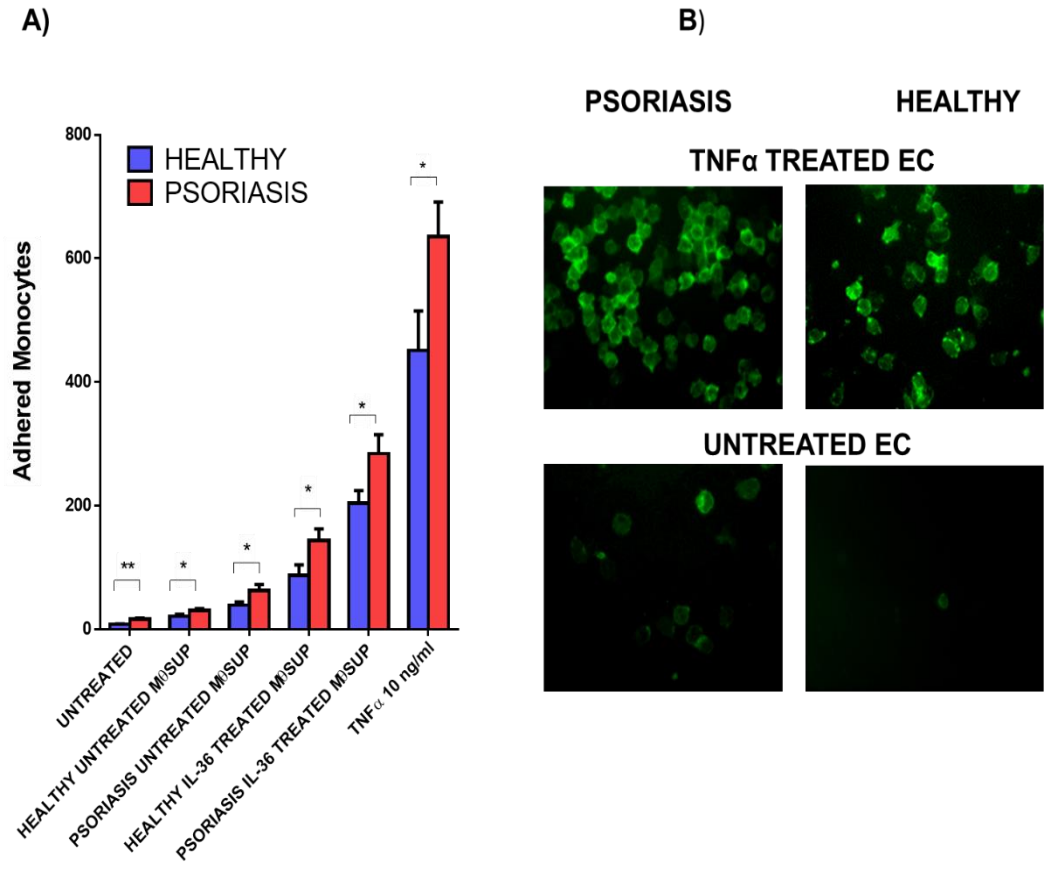

Figure 33: Psoriasis monocytes show increased adherence propensity

Both psoriasis and healthy monocytes were allowed to adhere to both treated (healthy and psoriasis macrophage supernatant, 24 hr) or TNFα (24 hr) treated HUVEC. After 30 mins, the number of adhered monocytes were counted. A) Number of adhered psoriasis and healthy monocytes per treatment. B) ICC showing adhered monocytes stained with anti-human CD14+ FITC. Magnification x 40. B) Psoriasis n=8 Healthy n=8. Unpaired t test *: p<0.05 from relevant controls.

3.3.7 CX3CL1 blocking has a greater effect on psoriasis monocyte adhesion

CX3CL1 selectively mediates CD16+ monocyte adhesion as opposed to CD14+. Psoriasis patients have elevated peripheral CD16+ monocytes and because psoriasis lesions show CD16+ migration, we proposed that blocking CX3CL1 would have greater effect on psoriasis monocyte adhesion when compared to healthy monocyte adhesion. HUVEC were stimulated with TNFα with and w/o an antibody blocking CX3CL1. Blocking CX3CL1 had a greater effect on psoriasis monocyte adhesion as proposed.

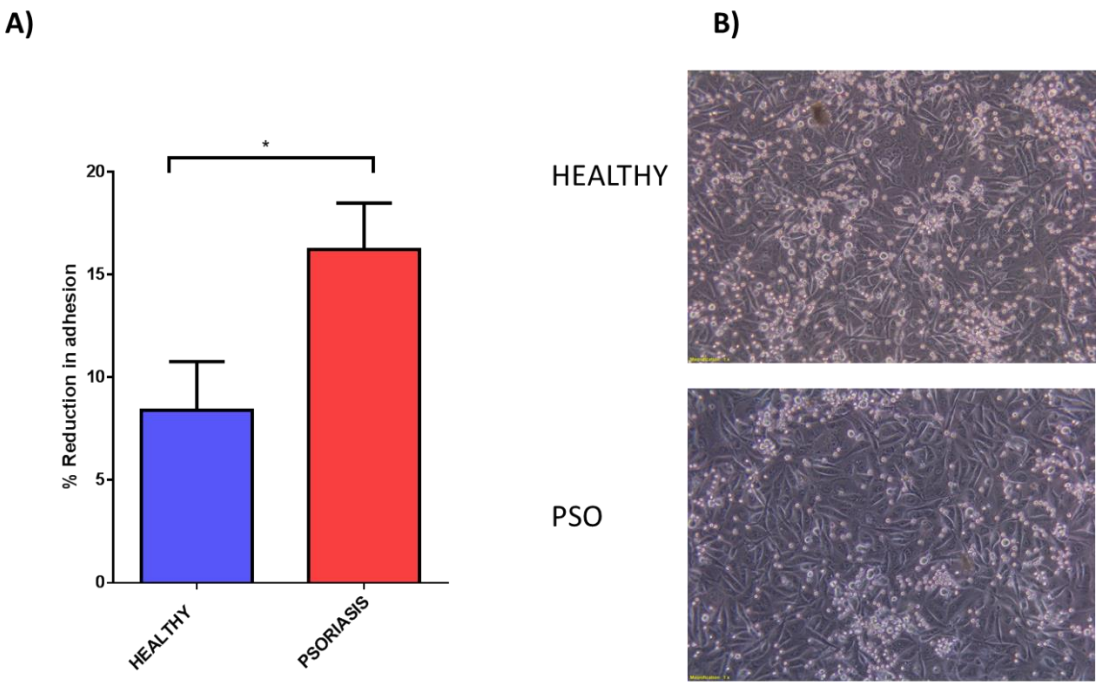

Figure 34: Blocking CX3CL1 has greater effect on psoriasis monocyte adherence

Psoriasis and heathy monocytes were allowed to adhere to TNFα stimulated HUVEC with and without CX3CL1 blocking. A) The number of

adhered monocytes were then counted and expressed as a percent reduction in adherence when compared to positive control (TNFα stimulated). Unpaired t test *: p<0.05. B) The number of adhered monocytes following CX3CL1 blocking was visualised for psoriasis and healthy monocytes.

3.4 Discussion

IL-36γ is an IL-1 family member cytokine with a growing importance in the pathology of psoriasis (D'Erme et al., 2015). Various myeloid cells are thought to contribute to the pathology of psoriasis, including resident macrophages (Clark and Kupper, 2006).

As IL-36γ stimulation of macrophages had not been fully characterised, we first established to confirm the presence of the IL-36R on macrophages (Figure 26). Once identified, the functional significance of the IL-36R was then confirmed by performing dose response curves to quantify TNFα secretion driven by IL-36 treatment of the macrophages. Following 48 hr stimulation, both 10 and 50 ng/ml of IL-36γ induced significant TNFα secretion from healthy macrophages, so these concentrations were chosen for future stimulations (Figure 27). IFNγ is known to activate macrophages, so this was investigated as a potential priming agent for IL-36γ 10ng/ml. Greatest statistical significance in TNFα induction by the macrophages was found when priming with IFNγ 20 ng/ml.

Myeloid cells, both infiltrating and resident are capable of secreting IL-23 and thus contributing to the IL-23/IL-17 axis, prominent in driving psoriasis (Zaba et al., 2009). Within psoriasis lesions, monocytes, M1 macrophages and dendritic cells all show positive staining for IL-23 (Yawalkar et al., 2009).

With IL-36γ being secreted by damaged keratinocytes, its downstream actions on resident myeloid cells such as macrophages could represent a key step in both early and chronic lesion pathology. While IL-36 has been shown to induce IL-23 secretion from dendritic cells, we report similar findings in macrophages (Vigne et al., 2011). Interestingly, other prominent inflammatory cytokines within the lesion, TNFα, IL-17 and IL-1, had little or no ability to induce IL-23 when compared to IL-36γ (Figure 29).

Psoriasis macrophages secreted significantly more IL-23 following IL-36γ stimulation than healthy patient macrophages. Our findings also compliment a recent mouse model of skin inflammation showing that overproduction of IL-36 by keratinocytes induces IL-23 secretion from macrophages, and that this is the driving force behind the pathology to a greater extent than IL-36 actions on dendritic cells (Ogawa et al., 2016). Moreover, the imiquimod induced mouse psoriasis model, is dependent on MyD88 signalling in macrophages and monocytes, hinting that TIR signalling as that which can be induced by IL-1 members is key (Costa et al., 2017).

IL-36γ-induced secretion of IL-23 was enhanced when macrophages were primed and activated with IFNγ. IFNγ enhancement of IL-23 secretion from macrophages has previously also been shown with other TIR agonists (van de Wetering et al., 2009, Butchar et al., 2008). IFNγ has also been shown to induce an inflammatory phenotype characteristic of psoriasis when injected into the skin and serum levels correlate with disease severity (Abdallah et al., 2009, Johnson-Huang et al., 2012). Within lesions this could also hypothetically be an IL-36 mediated effect given that IL-36 has been shown to induce IFNγ from CD4+ T cells (Vigne et al., 2012). Synergy with IFNγ was a prominent feature for IL-36

induced responses but not for IL-17, TNFα, or the similar IL-1 family member and TIR agonist IL-1. Previous reports have also shown that IL-1-induced TNFα secretion from macrophages is not enhanced by IFNγ (Ikejima et al., 1990).

TNFα was prominently induced in macrophages by IL-36γ (Figure 29), which is in line with a recent paper, showing IL-36γ upregulating TNFα gene expression in microglial macrophages (Bozoyan et al., 2015). Similar to the data on IL-23 production, psoriasis macrophages secreted significantly more TNFα when stimulated with IL-36γ and when untreated. IL-36γ induced TNFα from resident macrophages would be then well placed to potently stimulate the surrounding tissues to further orchestrate the immune response. Endothelial activation and thus increased leukocyte migration is central to lesion development. TNFα has a documented role within psoriasis immunology and several biologic treatments targeting TNFα have proved successful (Yost and Gudjonsson, 2009).

TNFα is known to be an inflammatory activator of the endothelium and we show that IL-36 induced macrophage supernatant is a potent activator of the endothelium, with the adhesion molecule ICAM-1 showing upregulation (Meekins et al., 1994) (Figures 30-32). In accordance with enhanced cytokine secretion seen from psoriasis macrophages, their supernatant was able to achieve increased endothelial activation when compared to healthy supernatant (Figures 31-32). Whilst, TNFα is a known activator of the endothelium, IL-36γ is likely to induce other cytokines from macrophages which could induce similar effects, so deciphering the most important activator of the endothelium within the supernatant requires future study.

Enhanced cytokine secretion from psoriasis macrophages is characteristic of the exaggerated immune response associated with psoriasis (Harden et al., 2015). Previous studies have also shown psoriasis macrophages secreting increased IL-8, IL-1α/β and TNFα at baseline, in agreement with our secretion findings (Okubo and Koga, 1998, Nishibu et al., 1999). Numerous mutations are thought to exist in psoriasis cells which lead to dysregulated immune responses (Harden et al., 2015). IL-36γ signals through NF-κB, and various mutations are thought to exist within psoriasis cells that lead increased activity of NF-κB such as CARD14 (Harden et al., 2015, Sun et al., 2013). Whilst damaged keratinocytes may be a potential source of IL-36γ, macrophages within lesions also show positive IL-36 staining, and lung macrophages are known to secrete IL-36γ in microparticles following LPS stimulation (Boutet et al., 2016, Kovach et al., 2016). Potential autocrine actions of IL-36 with macrophages thus require further study.

We report that following stimulation of HUVEC with IL-36-induced macrophage supernatant, monocytes show significantly increased adherence. Supernatant from psoriasis macrophages had increased ability to stimulate the endothelium, as quantified by ICAM-1 upregulation, and monocytes showed increased adhesion when compared to EC stimulated with healthy macrophage supernatant (Figures 31-33). Interestingly, regardless of the stimulus, psoriasis monocytes showed increased adhesion to both unstimulated and stimulated EC (Figure 33). Circulating monocytes in psoriasis patients are known to form aggregates with activated platelets which increases adhesion propensity of the monocytes to endothelial cells (Pamuk et al., 2009, Golden et al., 2015). Platelet binding also induces monocyte polarization to the inflammatory CD16+ subtype (Passacquale et al., 2011). Interestingly, platelet binding also enhances IL-23 secretion from

monocytes, while CD16+ monocytes have also shown to secrete increased levels of IL-23 (Scull et al., 2010, Rossol et al., 2012).

CX3CL1(fractalkine) is a chemokine that exists in both soluble and in a membrane bound forms and specifically mediates CD16+ monocyte transendothelial migration, as opposed to CD14+ (Ancuta et al., 2003). In psoriasis and other chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease, there is an elevation in peripheral CD16+ monocytes (Golden et al., 2015, Koch et al., 2010, Radwan et al., 2016). Thus, it could be hypothesised that blocking CX3CL1 would have greater efficacy on psoriasis monocytes due their increased numbers of CD16+. CX3CL1 is upregulated within psoriasis lesion endothelial cells and soluble CX3CL1 correlates with PASI (Congjun et al., 2015, Echigo et al., 2004). Psoriasis lesions also show heavy staining for CD16+ monocytes, indicating their transmigration to the tissues is key (Golden et al., 2015). In a recent imiquimod mouse model of psoriasis, mice with KO CX3CLR1 showed decreased dermal inflammation and this was attributed to a decreased in M1 macrophages (Morimura et al., 2016). An allele in CX3CR1 which results in leukocytes having less affinity for CX3CL1 is associated with a protective function in CVD (McDermott et al., 2003). This allele is statistically lower in psoriasis patients when compared to healthy controls, suggesting a possible reason for decreased CX3CL1-CX3CR1 interactions seen in healthy monocytes when compared to psoriasis (Plant et al., 2006). A monoclonal antibody targeting CX3CL1,KANAb001 (E6011), has shown positive results for treating RA and is currently at Phase II (Imai and Yasuda, 2016).

The findings presented here also may have implications for a host of other diseases. Crohn's disease shares many similar immunological aspects with

psoriasis, namely the IL-23/IL-17 axis, but also, a potential role for IL-36 in Crohn's disease is becoming apparent (Siakavellas and Bamias, 2012, Boutet et al., 2016). Interestingly, angiogenesis is also a feature of Crohn's disease (Alkim et al., 2015). Similarly, IL-36 has been implicated in mouse models of respiratory infection and again linked to the IL-23/IL-17 axis, and furthermore, angiogenesis is associated with chronic lung inflammation (Kovach et al., 2015, Kovach et al., 2017, Matarese and Santulli, 2012). Whilst IL-36 is yet to be fully implicated in COPD, cigarette smoke, the causative agent of COPD, induces IL-36 from bronchial epithelial cells (Parsanejad et al., 2008). COPD is heavily associated with Th17 cell driven inflammation (Alcorn et al., 2010). Psoriasis is emerging as a risk factor for COPD, and furthermore, mouse models of psoriasis show enhanced airway inflammation attributed to IL-23 signalling (Dreiher et al., 2008, Li et al., 2015, Nadeem et al., 2017). IL-36γ has recently been implicated in periodontitis, another disease in which the IL-23/IL-17 axis has gained momentum in recent years (Kurşunlu et al., 2015, Huynh et al., 2016, Ohyama et al., 2009).

Psoriasis is an independent risk factor for atherosclerosis (Miller et al., 2013, Ogdie et al., 2014). While the role of IL-36 in atherosclerosis development is presently unknown, potential effects based on our findings could be hypothesized. Macrophages and TNFα have an established role in atherosclerosis (Tam et al., 2014, Kleinbongard et al., 2010, Moore et al., 2013). In the vasculature, TNFα alters both the vascular smith muscle and endothelial function (Kleinbongard et al., 2010).

Recently, IL-23 has independently been associated with atherosclerosis disease progression with increased plasma levels correlating with mortality (Abbas et al.,

2015). The role of IL-17 in atherosclerosis remains controversial (Gong et al., 2015).

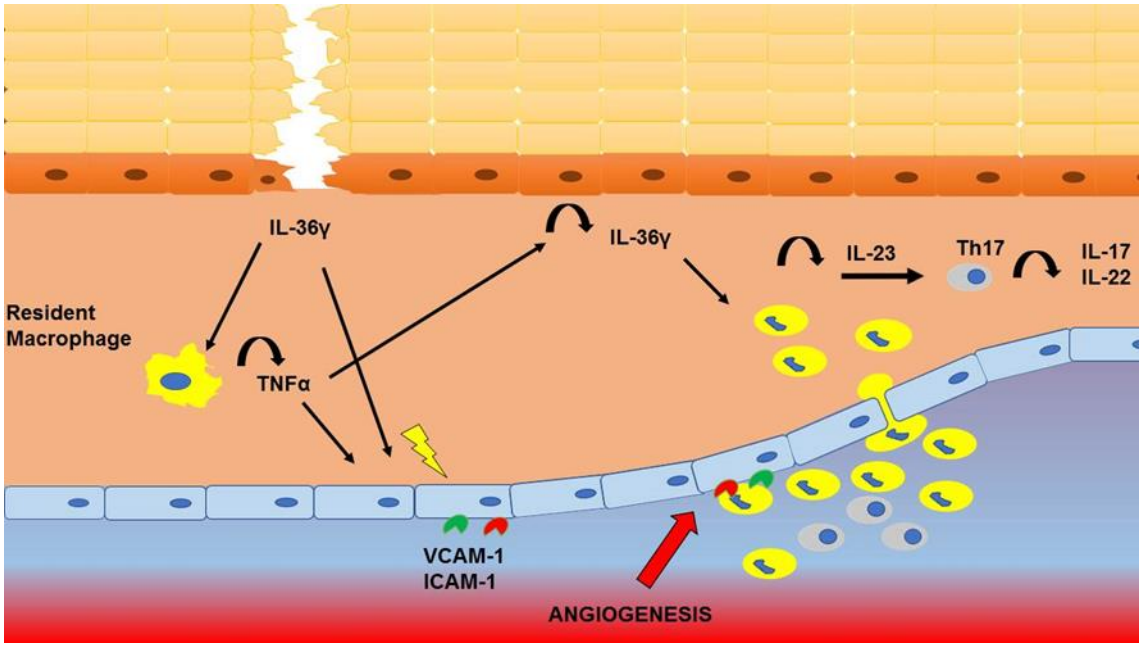

Figure 35: Proposed mechanism of IL-36 actions on monocytes in the dermal compartment

Injury in the skin leads to IL-36 secretion. IL-36 stimulates tissue resident macrophages. IL-36 induces TNFα from monocytes and macrophages. A combination of IL-36 and TNFα activate the endothelium and results in adhesion molecule/chemokine expression and angiogenesis. Circulating monocytes and T cells are recruited to the dermis and IL-36 induces IL-23 secretion from monocytes. IL-23 helps maintain the Th17 T cell phenotype typically found in psoriasis.

4) Endothelial progenitor cells as a source of psoriasis EC

Hypothesis

I hypothesise that similar to endothelial cells from psoriasis patients, endothelial cells derived from progenitor cells will have exaggerated immune responses, which could lead to enhanced atherosclerosis risk or development.

The vasculature is of central importance with respect to delivery, exchange, removal of gases, nutrients and regulatory cells to tissues and organs (Vanhoutte and Scott-Burden, 1994). Endothelial progenitor cells (EPC) have a role in regeneration of the endothelial lining of the blood vessels (Yoder, 2012). Since the discovery of novel endothelial progenitor cells populations 20 years ago, researchers have become interested in the therapeutic potential of these novel cells with respect to repair and regeneration of the systemic vasculature (Yoder, 2012).

EPCs can be isolated from both bone marrow and blood. Due to the difficulties in isolating mature ECs from within the blood vessels (patient subsets, lack of tissue, cost), the use of EPC from blood to study the function of ECs in certain disease subsets have become popular. Due to difficulties in acquiring psoriasis biopsies encountered within this research project, we attempted to isolate EPCs from consented psoriasis patients' blood samples.

However, the isolation of EPCs from blood has been met with numerous controversies over methodology and phenotype (Yoder, 2012). A definitive marker for EPCs is yet to be identified, and it is now thought previous studies which described EPC isolation from blood, may have described similar cells characteristically to EPCs, which will be described next, including the method we attempted.

4.1 Common methods to isolate EPC

4.1.1 Fibronectin coating

We decided to isolate EPCs using the fibronectin coating method, which has been previously described (Hill et al., 2003). Briefly, 15ml of blood was collected in heparin tubes. Blood was diluted 1:1 with PBS and layered over Lymphoprep density gradient medium by centrifuging at 800 x g for 20 mins. The mononuclear layer was subsequently isolated and PBMCs resuspended at concentration of 1 x 10^6 cells/ml in complete Promocell endothelial media described previously. Flasks were coated in fibronectin (Sigma) 1 µg/cm^2 and allowed to air dry overnight. PMBCs were then left to adhere for 48 hr and non-adherent cells were subsequently removed. Cells were then left for up to 30 days/ until EPC appeared with media being changed every 3 days.

Between 10-30 days, cells did not appear endothelial-like but more like macrophages in appearance. These cells had no proliferative capacity following passaging.

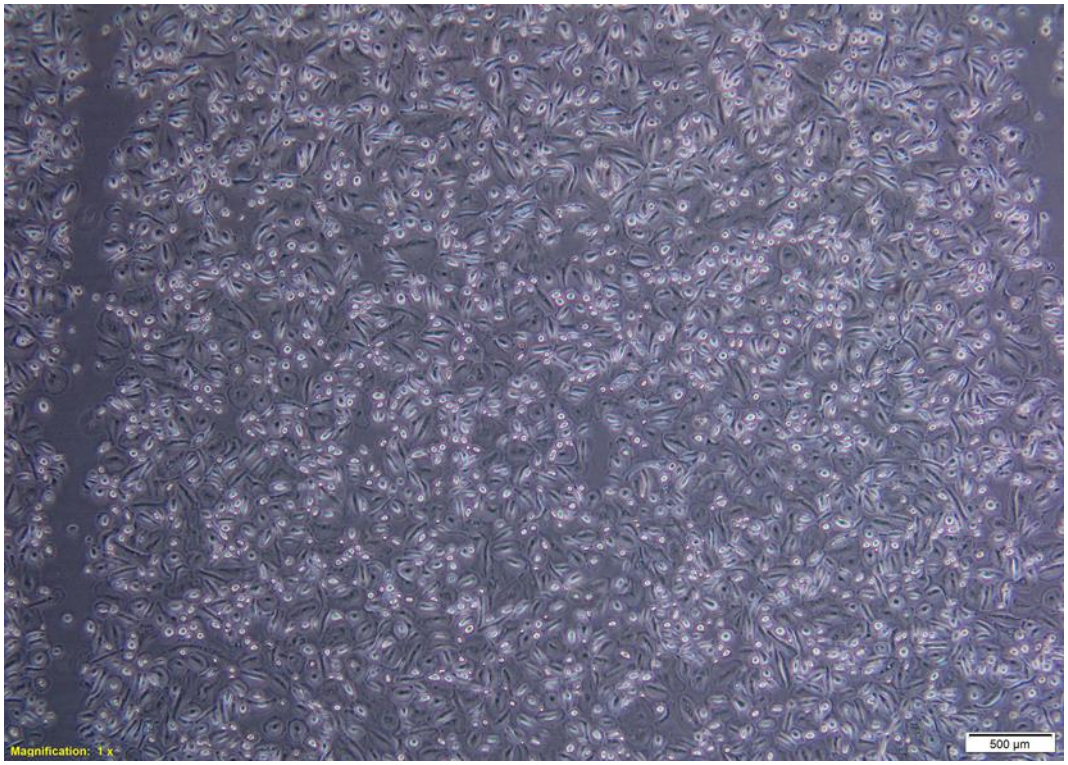

Figure 36: Isolated mononuclear cells plated on fibronectin

Isolated cells, with a macrophage-like appearance following 20 days of culture. Magnification x 20.

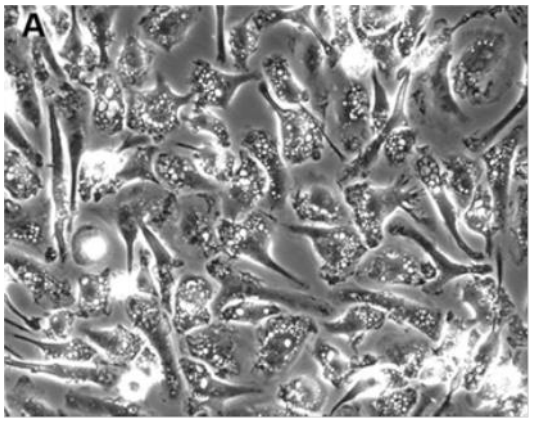

Figure 37: ***Example of macrophage morphology in long term culture, taken from (Anzinger et al., 2010).***

Despite others having claimed to have grown EPC from the fibronectin method there have been numerous controversies surrounding this method. Circulating

platelets are known to contaminate isolated PBMCs and release membrane particles that bind to the 'alleged' EPC. Platelet membranes and ECs have many surface proteins in common. It is thus possible that all adhered cells, including those of myeloid lineage could express EC proteins on their cell surface (Prokopi et al., 2009). Monocytes from PBMCs are also isolated from blood by binding to fibronectin. Monocytes cultured with endothelial growth factors such as VEGF express numerous EC surface proteins such as CD31 and vWF, and show eNOS upregulation (Schmeisser et al., 2003). It is now thought that EPCs isolated using the fibronectin method, resemble monocytes treated with VEGF and are now defined as 'circulating angiogenic cells' (CACs) (Rehman et al., 2003).

4.1.2 EPC isolation using FACS

Several surface markers have been proposed for circulating EPC. CD34 and CD133 have been used to isolate EPC, but the expression of these proteins is not restricted to EPC and are expressed by all hematopoietic stem and progenitor cells (Peichev et al., 2000). A number of hematopoietic stem and progenitor cell subsets have been described as displaying pro-angiognenic activity (Wara et al., 2011). Thus, cells isolated using cell sorting may also not be 'true' EPC.

4.1.3 Collagen method

A similar method to the previously described fibronectin method, substitutes type 1 collagen for fibronectin (Ingram et al., 2004). After 2-3 weeks, colonies of EPC begin to appear. These colonies have great proliferative capacity when compared to the other two methods (Yoder et al., 2007). EPC isolated using this method display all EC surface markers and have other EC characteristics such

as being able to form capillary-like structures when on Matrigel (Cheng et al., 2011, Melero-Martin et al., 2007).

4.2 Conclusion

The isolating and culture of EPC remains controversial, due to differing results in the literature over methodology. With respect to using EPC as a model to represent EC from diseased patients, this should thus be treated with caution.

5) Conclusions and future work

Endothelial cells play an essential role in tissue inflammation in diseases such as psoriasis. Endothelial cells and their activation is also crucial to the pathology of atherosclerosis. With psoriasis being an independent risk factor for atherosclerosis, research into endothelial function in psoriasis patients is of great importance as it could provide mechanistic information with beneficial therapeutic potential. The exact mechanisms of why psoriasis is a risk factor for atherosclerosis are still hotly debated. A combination of both systemic and genetic factors is likely to contribute to the increased risk. The data presented in this thesis and associated publications suggest that the novel cytokine IL-36 has a role in endothelial function and may contribute to psoriatic endothelial activation both directly and indirectly.

Conclusions:

- **The psoriasis relevant cytokine IL-36γ is an activator of both the macrovascular and microvascular human endothelium**

  This finding potentially could enhance psoriatic skin inflammation by enhancing immune cell recruitment. IL-36 activation of the vascular endothelium within vessels could also have importance in promoting atherosclerosis development and progression.

- **IL-36γ stimulated EC secrete CCL20 which recruits T cells**

CCL20 is a chemoattractant for T cells with a known function in psoriasis pathology. Endothelial cell expression of this chemokine may aid this chemotactic gradient.

- **IL-36γ is an activator of macrophages and induces inflammatory cytokine secretion such as IL-23 and TNFα, both of which are of pathological importance in psoriasis**

  Both IL-23 and TNFα have been shown to have pathological importance within psoriasis lesions and biological blockage of these molecules has proved successful for treating psoriasis symptoms. IL-36 is known to induce IL-23 from dendritic cells, but I am the first to report similar findings in macrophages. In accordance with this, macrophages within psoriasis lesions shown IL-23 staining. The IL-23/Th17 pathway is also starting to gain attention in atherosclerosis, which is of interest given the psoriasis-atherosclerosis link.

- **Other cytokines with known importance in psoriasis such as IL-17, TNF and IL-1 were unable to induce IL-23 from macrophages to the same degree as IL-36γ**

  TIR agonists such as LPS have a known function of upregulating IL-23 secretion, and TIR cytokine agonists such as IL-36 are known to upregulate IL-23 in dendritic cells. We report that other cytokines that are not TIR agonists, such as IL-17 and TNFα have no ability to induce IL-23, while similar family member and fellow TIR agonist IL-1 had reduced ability compared to IL-36γ. This highlights the potential role of IL-36 in regulating the IL-23/IL-17 axis.

- **Psoriasis macrophages show enhanced secretion of IL-23 and TNFα cytokines when compared to healthy**

  Many genetic predispositions that effect immune function are known to exist in psoriasis cells. We report that psoriasis macrophages secrete enhanced levels of inflammatory mediators when compared to healthy macrophages. Overexpression of these mediators is in line and characteristic of the over exaggerated immune response associated with psoriasis. Functionally, increased secretion of IL-23 could lead to increased maintenance of the Th17 pathway. As stated above, Th17 pathway is well documented in psoriasis, but also is a growing area of interest in atherosclerosis.

- **Psoriasis monocytes show increased adherence to both a stimulated and resting endothelium**

  Previous research has shown psoriasis patients' monocytes are activated and often form aggregates with other cells such as platelets, which primes them for activation. We report that psoriasis monocytes show increased adhesions to both resting and stimulated endothelium. This finding could have implications for both psoriasis in the form of the immune cell recruitment to the skin, but also atherosclerosis.

- **CX3CL1 blocking has a greater effect on psoriasis monocyte adhesion then healthy monocyte adhesion**

  Psoriasis patients have an elevation in CD16+ monocytes. CX3CL1 is a selective chemokine for CD16+ monocyte adhesion. Psoriasis plaques also show staining for CD16+ monocytes. We report blocking CX3CL1 reduces psoriasis adhesion significantly more than healthy monocyte

adhesion in line with the increased CD16+ population associated with psoriasis.

5.1 Future work

IL-36 in murine models of atherosclerosis

We have presented several possible potential mechanisms of how IL-36 may contribute to atherosclerosis. Both endothelial and macrophage activation represent key pathological components of the disease. A monoclonal antibody targeting IL-36 has long been suggested for treating psoriasis (Wolf and Ferris, 2014). It is thus crucial to explore the role of IL-36 in atherosclerosis murine models to determine its role if any in atherosclerosis. At present, links between IL-36 and other key components of atherosclerosis such as smooth muscle cells and foam cells are unknown.

Both IL-23 and IL-17 are starting to gain attention in regards to atherosclerosis, with paradoxical findings reported, given IL-36's relation with this axis, this research must receive attention. Despite both IL-17 and IL-23 having paradoxical findings reported in atherosclerosis, psoriasis patients on both inhibitors for these cytokines have had no abnormalities in cardiovascular events.

IL-36R 'knockout humans' and atherosclerosis

'Born in Bradford' is a community based initiative to exome sequence 2162 British Pakistani individuals. A recent study identified 6 individuals with a biallelic missense changes in the IL1RL1 (gene which encodes the IL-36R). Further studies confirmed this missense change to be deleterious (Mahil et al., 2017).

However, the 6 individuals had no history of abnormalities with respect to immune function. This suggests blockade of IL-36 could be a therapeutic target. It would be thus interesting to identify further individuals with non-functioning IL-36R and study epidemiology with respect to atherosclerosis.

DITRA and atherosclerosis

Whilst epidemiological research into psoriasis and atherosclerosis is well recognised, the CVD for specific psoriasis subtypes is under-researched. It is presently not known if GPP patients and more specifically DITRA have an elevated risk for CVD risk. This again would add to the knowledge regarding IL-36 and atherosclerosis.

Potential role of platelets and IL-36

Platelet activation and subsequent monocyte/platelet aggregates are known to exist in psoriasis peripheral blood (Golden et al., 2015). Platelets also have a documented role in atherosclerosis (Lievens and von Hundelshausen, 2011). Interestingly it is not known if platelets express the IL-36R of if platelets secrete IL-36. Platelets are known to express the similar IL-1 receptor and IL-1β has agonistic activating properties to platelets. Platelets also are a potential source of IL-1β (Brown et al., 2013). Thus, the potential relationship between IL-36 and platelets could be multifaceted.

Functional significance of psoriasis patients' endothelial cells

Numerous genetic predispositions that are linked to immune function are associated with psoriasis, and importantly, these are thought to lead to exaggerated inflammatory responses with psoriasis cells. Future work should

attempt to isolate endothelial cells from psoriasis patients and subject them to various models linked to atherosclerosis to put the CVD risk into context.

IL-36, DITRA and diabetes

Diabetes is as a result of endothelial dysfunction (Tabit et al., 2010). Systemic cytokine stimulation is thought to cause endothelial dysfunction. With psoriasis being associated with diabetes, there would be also great value in study mechanisms behind IL-36 and diabetes and also the epidemiology of DITRA and diabetes.

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